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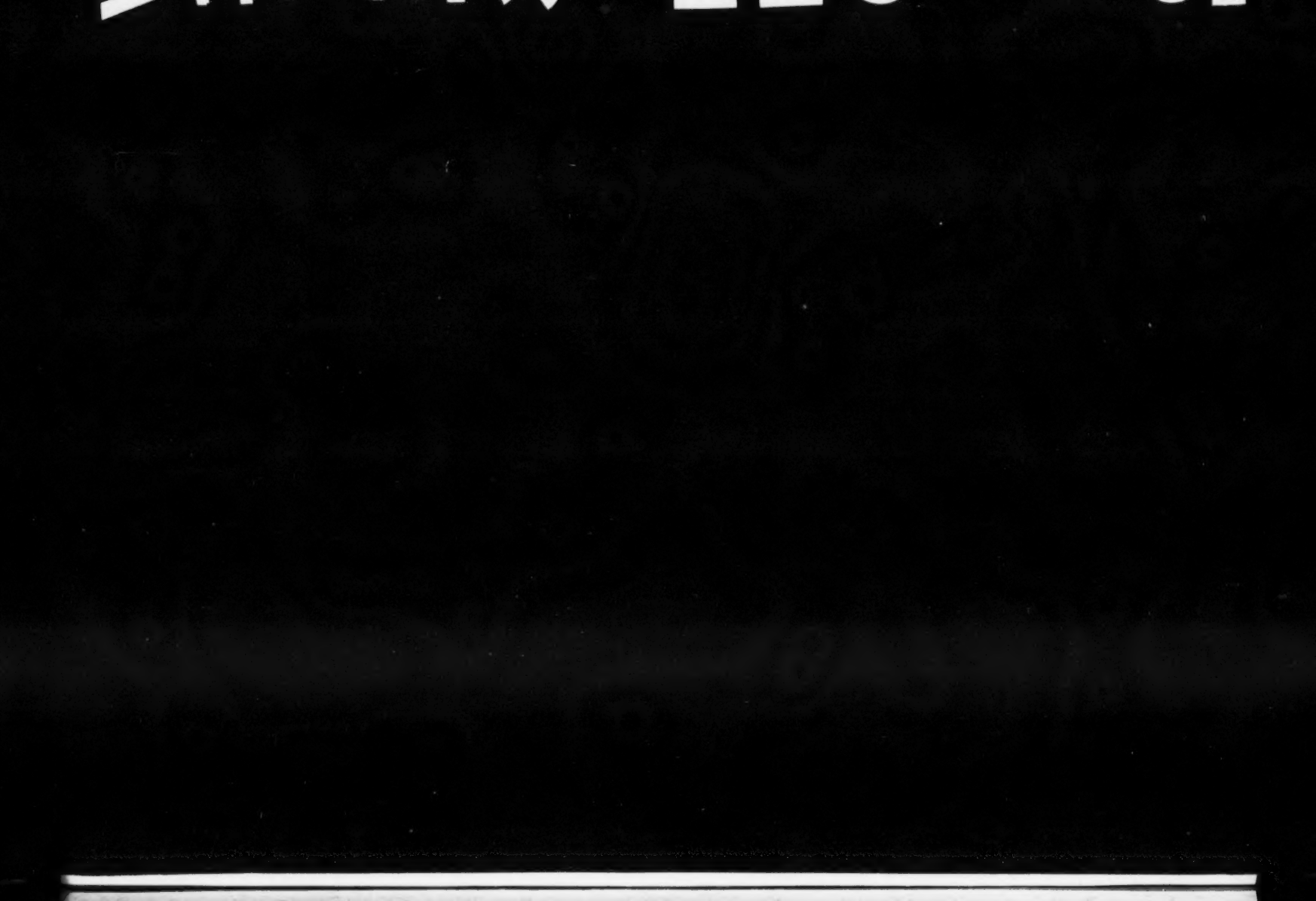

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THE
BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

A NEW PHYCOERYTHRIN FROM PORPHYRA NAIADUM [1]

R. L. AIRTH [2] AND L. R. BLINKS

Hopkins Marine Station of Stanford University, Pacific Grove, California

Svedberg and Katsurai (1929) proposed a phylogenetic nomenclatural system for the classification of the phycobilin pigments of the algae. They designated the phycoerythrin and phycocyanin from the red algae as R-phycoerythrin and R-phycocyanin, respectively, and the corresponding pigments from the blue-green algae as C-phycoerythrin and C-phycocyanin. In general, these pigments exhibit the following absorption maxima:

Pigment	Approximate absorption maxima mμ			
R-phycoerythrin	495	540	560	
C-phycoerythrin		550		
R-phycocyanin		550		615
C-phycocyanin				615

This system has proved inadequate in several instances (Kylin, 1912; Lemberg, 1930; Kylin, 1940; Haxo, *et al.*, 1955) in that phycobilin pigments other than the above types, as judged from their absorption spectra, have been isolated. In spite of the apparent shortcomings of the Svedberg and Katsurai system of classification, no new system has been proposed.

A new phycoerythrin has now been isolated from *Porphyra naiadum* and it is proposed that this pigment be called B-phycoerythrin (tentatively so designated by Blinks, 1954). This differs from known phycoerythrins in having two absorption peaks, at 545 and 565 mμ. The isolation, purification and some properties of this pigment will be discussed.

Source

Porphyra naiadum Anderson is a member of the most primitive red algal order, the Bangiales. There is now some question as to whether it belongs in the genus *Porphyra*, since its life cycle is different. This is currently under study by Prof. G. J. Hollenberg; pending his description of a new genus, we must use the current name. The thallus is one cell thick, extremely delicate, and yields its pigment readily into fresh water in a few hours. It is found growing only upon a marine flowering plant, *Phyllospadix*.

[1] Research supported under contract with the Office of Naval Research (No. NR 120-050).

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Materials and Methods

Masses of thalli were stripped from the host plant and washed with distilled water. The washed algal mass was then just covered with distilled water and kept at 5° C. for about 15 hours. The supernatant, which contains the water-soluble phycobilin pigments, was separated from the algae by centrifugation at 15,000 times gravity for one hour. By this procedure approximately 75 per cent of the total phycobilins present can be extracted. The supernatant was filtered twice through Whatman No. 1 filter paper and the filtrate centrifuged at 20,000 *g* for twenty minutes. The pH of the pigment solution at this stage was 6.8–7.0. The pigment solution was then dialyzed in "Visking" tubing for 12 hours at 1° C. against 0.1 *M* acetate buffer, pH 5.0. The dialyzed pH 5.0 pigment solution constituted the stock solution and will, in the future, be referred to as such.

Results and Discussion

Purification and crystallization

The general method of purification and crystallization of phycobilin pigments, which has varied slightly from investigator to investigator, involved precipitation with ammonium sulfate (Kylin, 1912; Kitasato, 1925; Lemberg, 1928). This precipitation is carried out after the algae have been extracted for several days at room temperature under slightly basic conditions.

This procedure was attempted on freshly extracted stock pigment solution from *P. naiadum:* it consistently failed to crystallize phycoerythrin and phycocyanin although concentrated ammonium sulfate precipitated an amorphous mass. If the stock pigment solution was allowed to stand at room temperature for several days, then phycoerythrin (but not phycocyanin) could be crystallized by this method. Bannister (1954) working with the blue-green alga *Synechocystis* apparently encountered similar difficulties in trying to crystallize with a freshly extracted pigment solution. It seems very likely that the phycoerythrin obtained by the classical procedure may be a modified pigment.

In contrast to ammonium sulfate treatment a freshly prepared stock pigment solution yielded well-formed phycoerythrin crystals on simply standing, in the cold, for about 24 hours at pH 4.5 These crystals were separated by centrifugation, washed in acetate buffer at pH 4.5, and redissolved in water adjusted to pH 7.5. Recrystallization could be carried out by reacidification to pH 4.5. The first crystallization gave an estimated 20 per cent yield of the total phycoerythrin present, while the second and subsequent recrystallizations were quantitative. The reason for this low yield in the original crystallization will be discussed in a subsequent communication. (Suffice it to say here that the non-crystallizable fraction appears to have an iso-electric point (if at all) in very acid ranges.)

The absorption spectra of the original crystals and three-times recrystallized B-phycoerythrin are presented in Figure 1. The main absorption maximum is at 545 mμ; this value did not vary between pH 5.0 to 7.0. Phycoerythrin that is kept at pH 9.0 for 6 hours has the same absorption maximum but the blue (400–450 mμ) and red (600–700 mμ) absorption is increased by about 300 per cent. Recently Haxo *et al.* (1955) have found that the phycoerythrin from *Porphyridium* was modified above pH 7.3. Our findings are also consistent with theirs in that

we find that phycocyanin from *P. naiadum* is irreversibly bleached at pH 9.0. In contrast to the phycoerythrin from *Porphyridium*, however, neither the native nor the crystalline B-phycoerythrin from *Porphyra naiadum* tended to form additional "shoulders" or absorption maxima under alkaline conditions.

In addition, it is apparent that the original B-phycoerythrin crystals exhibit a "shoulder" in the 565 mμ spectral region which is lacking in the three-times recrystallized preparation. Whether this absorption represents an impurity in the original crystallization or a modification of phycoerythrin on repeated recrystalliza-

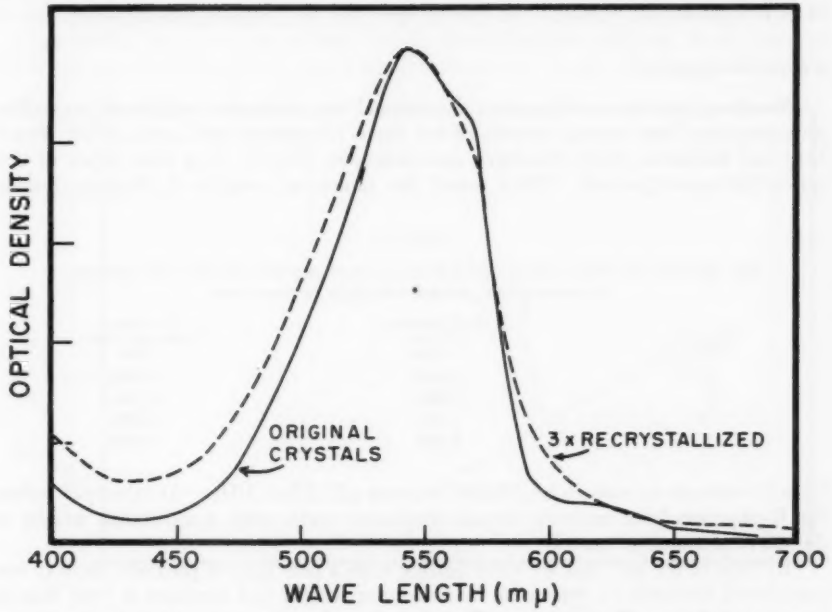

FIGURE 1. Absorption curves of once- and three-times recrystallized B-phycoerythrin. Solid line, original crystals; dashes, three-times recrystallized.

tion is unknown at the present time. There are, however, reasons for believing that the latter may be the case. On repeated recrystallization it was found that the B-phycoerythrin solubility at pH 7.5 decreased with the number of times the pigment was recrystallized. Also the stability of the native and crystalline phycoerythrin to hydrogen peroxide is different. Table I gives the reduction in optical density at 540 mμ of the two pigment solutions when treated with varying hydrogen peroxide concentrations. The pH of both solutions was 5.8.

B-phycoerythrin was also isolated by the chromatographic method of Swingle and Tiselius (1951). Haxo *et al.* (1955) have successfully separated the phycobilin pigments, including allophycocyanin, from several algal species by this method. The latter pigment is also present in *P. naiadum* and this was the only method we found to isolate allophycocyanin and phycocyanin in relatively pure form.

The order of pigment elution from the column depends upon the pH of the elutant. Using 1 to 2 *M* acetate buffer at pH 5.0 as an elutant, the pigments come off the column in the following order: phycocyanin, B-phycoerythrin, a mixture of phycoerythrin and phycocyanin. Allophycocyanin could not be eluted from the column at pH 5.0; however, it was eluted with 0.1 *M* phosphate buffer, pH 7.0. When phosphate buffer (0.05 to 0.1 *M*), pH 7.0, was used the elution order of the pigment was: B-phycoerythrin, a mixture of phycoerythrin, phycocyanin and allophycocyanin, and finally allophycocyanin.

The phycoerythrin isolated by this method was, however, never completely free of phycocyanin and repeated chromatography did not remove this impurity.

Ultracentrifugation

Svedberg and his collaborators determined the molecular weight of crystalline phycoerythrin from several species of red algae (Svedberg and Lewis, 1928; Svedberg and Katsurai, 1929; Svedberg and Eriksson, 1932). In a final paper of this series Eriksson-Quensel (1938) found the molecular weight of R-phycoerythrin

TABLE I

The reduction in optical density (at 540 mμ) of stock pigment solution and crystalline B-phycoerythrin, treated with H_2O_2 for four hours

Per cent H_2O_2	Stock pigment solution $-\Delta E_{540}$	Crystalline B-phycoerythrin $-\Delta E_{540}$
0	0.040	0.000
1	0.874	+0.204
3	1.146	0.000
5	1.060	0.000

from *Ceramium rubrum* to be 290,000 between pH 3.0 to 10.0. At other pH values the R-phycoerythrin molecule breaks down into units with a molecular weight of 34,600 or a multiple thereof.

In view of the fact that we were dealing with a new type of phycoerythrin it was considered desirable to determine its molecular weight and compare it with that of R-phycoerythrin. The ultracentrifugation experiments were carried out for us through the very kind courtesy of Drs. H. Cook and J. M. Luck of the Department of Chemistry, Stanford University.

B-phycoerythrin that had been recrystallized several times was dissolved at pH 7.0, centrifuged, and dialyzed at 1° C. in 0.05 *M* acetate buffer, pH 5.0. The sedimentation constant ($s_{20} \times 10^{13}$) was determined on the dialyzed preparation in a Spinco ultracentrifuge and found to be 12.0. This value is comparable to that found by Eriksson-Quensel (1938). Consequently it seems very probable that the molecular weights of R- and B-phycoerythrin are the same. The crystalline B-phycoerythrin was judged to be homogeneous from the fact that only one schlieren peak was observed and the absorption boundary of the pigment corresponds almost exactly with it. It may also be noted that Svedberg and Eriksson (1932) determined the molecular weight of what they designated native R-phycoerythrin from *Ceramium* and found it to be similar to the crystalline phycoerythrin from this species.

Electrophoresis

The mobility of crystalline B-phycoerythrin was tested by dissolving it at pH 7.5, centrifuging and then dialyzing at 1° C. in acetate buffer of 0.1 ionic strength, pH 5.0. The movement is toward the anode at the rate of about 2.0×10^{-5} cm.^{2}/sec./volt.

The above value was determined by visually measuring the movement of the ascending and descending boundary in a Tiselius apparatus (no schlieren optics were available). Fairly reproducible results could be obtained, because of the intense color of the pigment.

Crystalline B-phycoerythrin had charge characteristics different from those of the remainder of the pigments in the stock solution and it was possible to determine

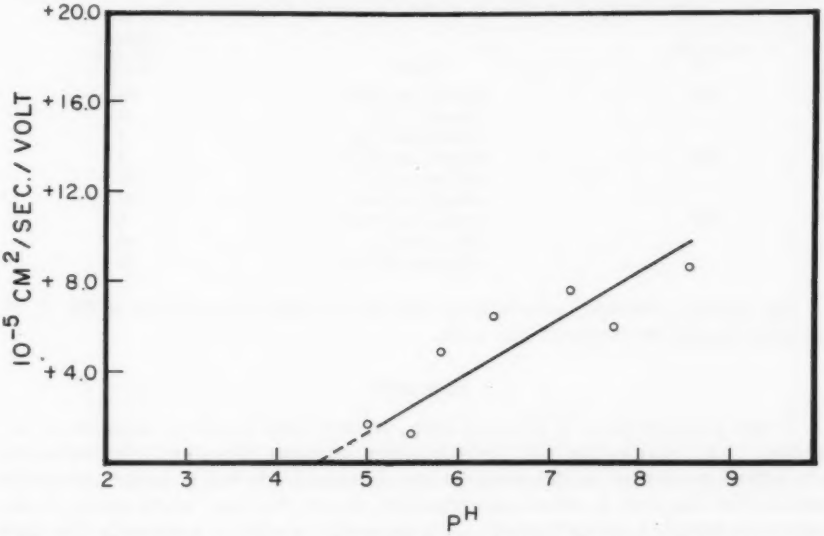

FIGURE 2. pH-mobility curve of crystalline B-phycoerythrin. Mobility in 10^{-5} cm.^{2}/sec./volt. Determinations by visual measurement in Tiselius apparatus.

the pH-mobility characteristics of this pigment. In this case the mobility values were determined from either the ascending or descending boundary, depending upon the pH of the determination. The pH-mobility curve of B-phycoerythrin is presented in Figure 2. No values were determined below pH 5.0 as the pigment tended to precipitate below this pH value. The isoelectric point as extrapolated by this method would be approximately at pH 4.5 which corresponds well with that found for crystallization.

Other optical properties

The fluorescence spectrum of B-phycoerythrin has been determined by French, Smith, Virgin and Airth (unpublished data); at pH 7.0 the fluorescence maximum is at 578 mμ.

The phycobilin constituents of *P. naiadum* are B-phycoerythrin, phycocyanin and allophycocyanin. At least the two former pigments are photosynthetically active in that they pass absorbed light energy on to chlorophyll. In such studies it is often essential to know the percentage of the total light absorbed by each pigment at various wave-lengths. As purified pigments were available a curve analysis of the absorption of the stock pigment solution was carried out. The percentage of the total light absorbed by each of the phycobilin pigments at their respective absorption maxima is presented in Table II. These values are fairly consistent with those presented by Yocum and Blinks (1954).

TABLE II

Percentage absorption of the total light absorbed by the various phycobilin pigments at different wave-lengths of an extracted pigment solution of Porphyra naiadum

Wave-length mμ	Pigment	Per cent total light absorbed
545	B-phycoerythrin	88
	phycocyanin	9
	allophycocyanin	3
615	B-phycoerythrin	2
	phycocyanin	77
	allophycocyanin	21
655	B-phycoerythrin	0
	phycocyanin	18
	allophycocyanin	82

The authors gratefully acknowledge the many helpful suggestions of Dr. C. B. van Niel during the course of this work.

SUMMARY

A new phycoerythrin, B-phycoerythrin, isolated from *Porphyra naiadum*, is described. The purification and crystallization, ultracentrifugation, electrophoretic and optical properties of this pigment are discussed. It has a major absorption peak at 545 mμ with a minor, and transient, one at 565 mμ, which tends to disappear on repeated crystallization. The molecular weight is apparently the same as that of R-phycoerythrin (ca. 290,000). Its iso-electric point is close to pH 4.5, and its mobility (toward the anode) at pH 5.0 is about 2×10^{-5} cm.²/sec./volt.

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HERMAPHRODITISM IN ECHINOIDS[1]

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In his chapter on "Hermaphroditism" Goldschmidt (1923, p. 165) speaks of this as "the most unsatisfactory chapter in the whole sex problem, and up to date our material is insufficient to permit of a correct genetic or physiological understanding." The problem is still further rendered difficult by the fact that it cannot be dealt with experimentally. In the echinoids hermaphroditism, as a rule, is shown by entire gonads of an individual being of one sex or the other, *i.e.*, testes and ovaries in the same animal. More rarely ovarian and testicular tissues develop side by side in the same gonad—an ovotestis. In the five gonads radially symmetrically disposed, all possible combinations have been found, the rarest being the ovotestis.

Such a condition is of sufficiently infrequent occurrence to be noted in the literature in a number of cases in which it has been observed. The paucity of known cases is well expressed by E. B. Harvey (1939, p. 74): "Among the many thousands of *Arbacia punctulata* opened in the course of ten summers at Woods Hole, and many hundreds of *Arbacia pustulosa, Sphaerechinus granularis, Paracentrotus lividus,* and *Parechinus microtuberculatus* opened during several springs at Naples, and many hundreds of *Strongylocentrotus droebachiensis* from Maine, I observed last summer for the first time an hermaphroditic sea urchin, an *Arbacia punctulata* opened on July 4, 1938."

This situation renders an analysis of the phenomenon difficult, and the impossibility of attacking the problem experimentally prevents a precise causative analysis. Nevertheless, an examination of the cases described, the frequency of their incidence geographically and in the classification may give some clue to the phenomenon of what may be called "accidental hermaphroditism." It is hoped that this collection of records may serve to stimulate an interest in the problem and result in further information being published.

At the present time very few statistical records of bisexual echinoids are available. H. B. Moore (1932) reported one hermaphrodite in 3000 *Echinus esculentus* opened during the season 1931–32 at Port Erin. Shapiro (1935) kept an exact account of the *Arbacia punctulata* which he opened during the summer of 1935. He found one hermaphrodite in 2350 animals opened. Albert Tyler (personal communication) at Corona del Mar has kept the most extensive records and reports that 10,000 *Strongylocentrotus purpuratus* opened over a period of several years yielded approximately 20 hermaphrodites, or 1 in 500. This is the highest incidence so far reported. Tyler found that, in addition to normal development as the result of selfing, agglutination of the sperm by autologous sea water was positive. Edward Chambers, working at Berkeley with *S. purpuratus,* in the course of two seasons observed three hermaphrodites.

[1] This work was supported in part by a grant from the National Science Foundation and Grant B-160 (C) from National Institutes of Health, Public Health Service.

At Pacific Grove, although the use of sea urchins has gone on for many years, only one bisexual individual each of *S. purpuratus* and *S. franciscanus* has been detected. In all these cases the selfed eggs gave normal plutei.

In the specimen of *S. purpuratus* opened at Pacific Grove in December, 1950 (A.R.M.), the gonads were swollen with ripe products and easily broken. There were three ovaries, one testis and one ovotestis. None of the eggs could have been fertilized *in corpore*, since no fertilized or segmenting eggs or embryos were seen at the time the animal was opened. However, as soon as the eggs and sperm were free in the sea water, fertilization took place. Development of the selfed eggs was entirely normal, the plutei differing in no way from normals.

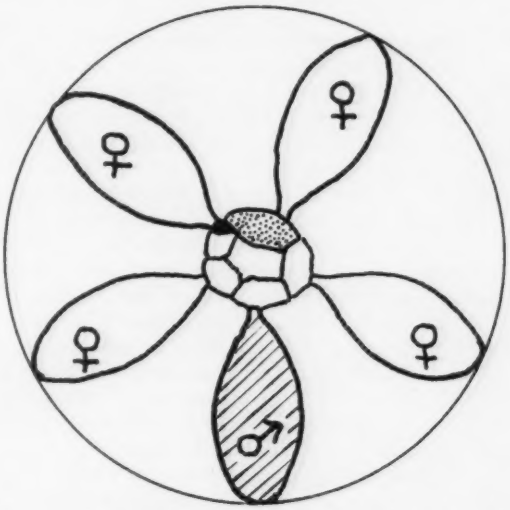

FIGURE 1. Topographical scheme showing details of female and male gonad elements of *S. franciscanus*, viewed from the aboral surface.

In March, 1955, a bisexual individual *S. franciscanus* was found (R. A. B.) at Pacific Grove. This was the first such individual of this species to be discovered. The gonads were separate as to sex, four ovaries, one testis, and no ovotestis. The distribution of the gonads is shown in Figure 1. The selfed eggs produced normal plutei as did the outcrossing of both eggs and sperm. It is to be noted that this is the first case of bisexuality found in this species. The very short breeding season may be a factor.

In addition to these examples of sea urchins, several hermaphroditic individuals of *Dendraster* have been found at Pacific Grove. The first was in 1929 (Needham and Moore, 1929), when hundreds of the animals were being used to obtain material for chemical work. In every case the entire gonadal disk was removed and any disk containing white sperm and red eggs would have been detected at once. Since only one was found, it may be assumed that the incidence was of the order of one in 1000. In this one case the eggs and sperm were not fertile *inter se*. The

second specimen of *Dendraster* was taken in 1943, and contained both ripe sperm and eggs. The spermaries occupied a little more than half the gonadal disk and yielded abundant sperm. The ovarian half contained a few ripe eggs. Less than half of these were fertilizable with the sperm of the same individual. Such eggs segmented at the normal cleavage rate and gave rise to swimming blastulae in normal time, but the plutei were not vigorous, some larvae remaining blastulae. At 55 hours and 20° C. these selfed larvae were in all stages of juvenility. It is evident that the eggs were defective in their potential, but the sperm was normal since it brought about normal development in normal eggs.

The Ovotestis

The occurrence of eggs and sperm in the same gonad has been observed in several widely different groups of animals and is a type of accidental hermaphroditism according to Goldschmidt. Ishikawa (1891) described a case in *Gebia major* in which the anterior part of the gonad was testis, the posterior part ovary. The latter was not functional since the eggs were unable to pass out through the vas deferens and consequently atrophied *in situ*. Paul Buchner (1911) described in careful detail the gonads of a bisexual starfish. He observed eggs about the sperm in the testicular vesicles, and ripe sperm infiltrated into the ovary. Harvey (1939) has shown similar conditions in a hermaphrodite *Arbacia*. All of the gonads contained both types of cells, four being mainly female, one predominantly male. Therefore all five gonads were ovotestes. Normal fertilization took place *inter se* and development proceeded to normal plutei. Similar instances have been described by Neefs (1953) and by Reverberi (1947). An account of a hermaphroditic sea urchin *S. pulcherrimus* has been published by Okada and Shimoizumi (1952), in which they give a very complete analysis. One gonad was an ovary, the others were ovotestes. The eggs and sperm did not yield normal larvae when used *inter se*, but gave normal larvae on out-crossing.

As stated above, the hermaphroditic *S. purpuratus* found at Pacific Grove contained one ovotestis. This gonad was preserved and imbedded by Dr. D. P. Abbott at this station. The specimen remained in block until the present season when it was sectioned and mounted by Mr. W. K. Bowen of the Biology Department of Stanford University. The gonad appeared to be divided into an upper and lower half which were, respectively, ovary and testis. Sections were made of parts that were clearly unisexual and of the mixed median zone. Sections of the ovarian half show the ovarian lobes well filled with eggs both ripe and immature (Fig. 2), while sections of the testicular half show normal testicular structure and dense collections of sperm in the vesicles and ducts (Fig. 3). In the median section ovarian and testicular tissue lie side by side, the acini intermingled in the same section (Fig. 4). Ripe ova occur among the sperm (Fig. 5), but no eggs were found fertilized, a fact which presumably was due to the immobility of the sperm. This duplicates the situation in the case described by Harvey.

Two clear exceptions to the general rule that fertilization does not occur *in corpore* before the extrusion of the sex cells from an ovotestis have been noted. H. B. Moore (1935) describes one such case in *Echinocardium cordatum*. In his sketches he figures apparently normal early segmentation stages, morulae and blastulae present in the gonadal ducts of the ovotestis. Reverberi (1947) has described

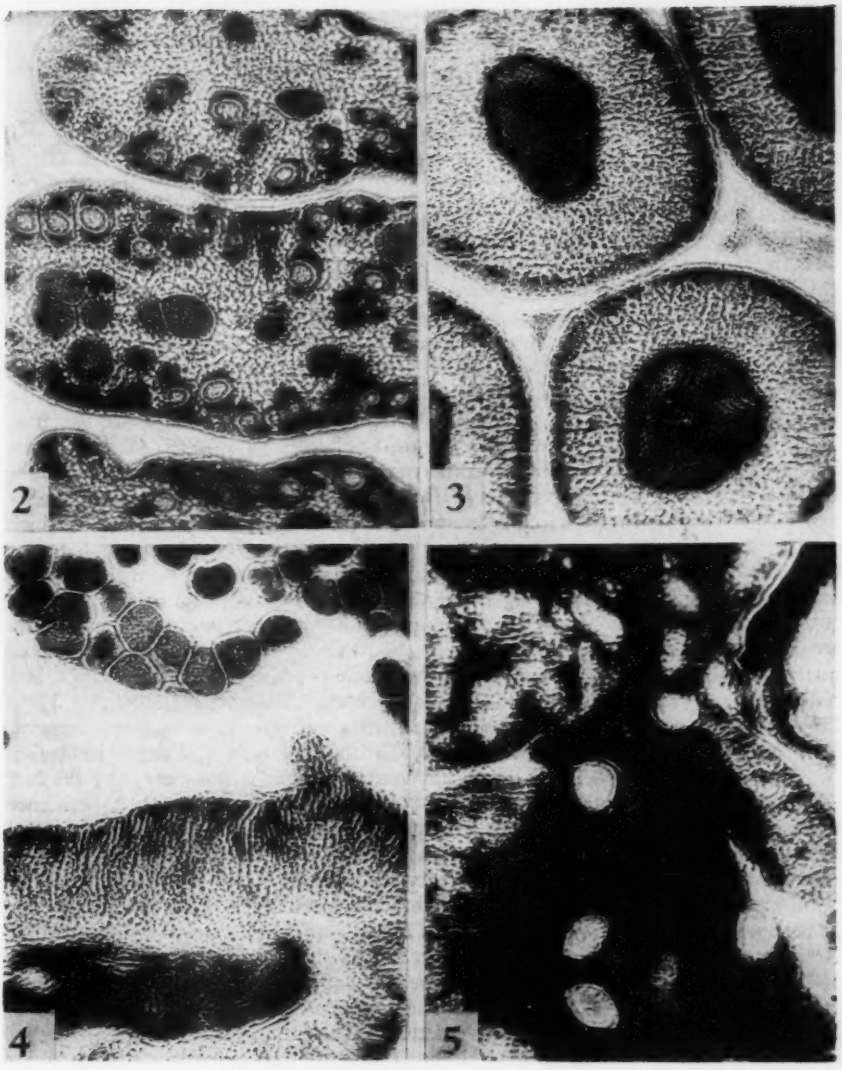

FIGURE 2. Ovarian fraction of ovotestis.
FIGURE 3. Testicular fraction of ovotestis.
FIGURE 4. Boundary zone showing ovarian tissue above, testicular below.
FIGURE 5. From boundary zone showing vesicle with eggs in mass of sperm.

a similar case in *Arbacia pustulosa* at Naples. However, Neefs' (1953) figure of a cell-mass in the lobe of an ovary of a bisexual *Arbacia lixula* is of doubtful significance, for the reason that the structure appears to be a relatively unorganized mass of cells without recognizable embryonic form, and is apparently a solitary instance in the specimen.

Discussion

Various causative factors have been proposed to account for hermaphroditism in echinoids. The oldest is the suggestion of seasonal dimorphism by Giard (1900) who, working at Wimereux, found evidence which he thought sufficient for concluding that *Echinocardium cordatum* is normally a protandrous hermaphrodite, for the reason that in July ova begin to appear in individuals which up to that time he believed had been male. However, Giard's conclusions were later emphatically denied by Caullery (1925) who states that the gonads of this form at Wimereux are entirely quiescent during autumn, the gametes developing during the winter, the phase of maturity beginning in April and ending in August, with a maximum in May. As a result of numerous observations, Caullery says (p. 29): "I have never found a single case of hermaphroditism (in this form) and I cannot explain how Giard could think that at Wimereux, *Echinocardium* shows successive sexuality with protandry, the eggs beginning to appear toward mid-July in the gonads which up to that time were apparently male and full of sperm." Since Caullery, the eminent zoologist and director of the Wimereux Station, has written from the vantage point of 25 years after Giard's paper, it must be considered established that Giard observed a rare case of hermaphroditism in *Echinocardium* (two others have been recorded at Roscoff and one at Port Erin), and that he was clearly in error in postulating seasonal sexual dimorphism for the whole population of this species. Recently Reverberi (1940, 1947) in Italy and Neefs (1937, 1938, 1952, 1953) in France have sought to revive Giard's hypothesis and give it substantial support. Reverberi considers the fact of an ovotestis in itself to be a significant indication of sexual metamorphosis. But to the unbiased worker it is not clear why the fact that normal eggs and sperm occur side by side in an ovotestis, with no evidence whatever of either type of gland degenerating, should indicate a process of sexual metamorphosis. It must be confessed that Reverberi's observations do not give convincing basis for his hypothesis. Neefs has used two lines of argument for her belief in the seasonal change of sex as the basis of bisexuality. One is the presence of degenerate gonads of two colors in an animal. Such a case was described by Gray (1921). We occasionally find them among the *S. purpuratus*. Since these pathological individuals often do not have either eggs or sperm, the more conservative view should be taken, namely, that the appearance is an indication of disease. The other attempt of Neefs to show seasonal sexual dimorphism has been made by means of statistical counts of sex. It must be said that her tables are not convincing, for the reason that at each station a relatively limited number of individuals was examined, and animals of both sexes appeared in each month of the year—a very different picture from that given by Giard. It should also be noted that no one except Giard has reported seasonal incidence of bisexuality.

Recently Egami (1955) has proposed a nutritional basis for bisexuality in fishes. He has shown that periods of starvation, succeeded by food in plentiful

TABLE I

Table of reported cases

Author	Species and cases	Locality
Normal Eggs and Sperm		
Boolootian (this paper)	*S. franciscanus*	Pacific Grove
Chambers (personal communication)	*S. purpuratus*	Berkeley
Cornman (Harvey, 1956)	*Arbacia punctulata*	Woods Hole
Fisher (Harvey, 1956)	*Arbacia punctulata*	Woods Hole
Fox (Gray, 1921)	*Paracentrotus lividus*	Naples
Giard (1900)	*Echinocardium cordatum*	Wimereux
Harvey (1939)	*Arbacia punctulata* (2)	Woods Hole
Herbst (1925)	*Psammechinus tuberculatus*	Naples
Heilbrunn (1929)	*Arbacia punctulata* (2)	Woods Hole
Herlant (1918)	*Paracentrotus lividus* (12)	Villefranche
Moore, A. R. (this paper)	*S. purpuratus*	Pacific Grove
Moore, H. B. (1932)	*Echinus esculentus*	Port Erin
Moore, H. B. (1935)	*Echinocardium cordatum*	Port Erin
Neefs (1938)	*Paracentrotus lividus* (7)	Roscoff
Neefs (1952)	*Sphaerechinus granularis*	Roscoff
Neefs (1937 and 1953)	*Arbacia lixula* (2)	Banyuls
Reverberi (1940)	*Arbacia pustulosa*	Naples
Tyler (personal communication)	*S. purpuratus* (20)	Corona del Mar
Defective Eggs—Normal Sperm		
Drzewina and Bohn (1924)	*Paracentrotus lividus*	Roscoff
	Echinocardium cordatum (2)	Roscoff
Moore, A. R. (this paper)	*Dendraster excentricus*	Pacific Grove
Normal Eggs—Defective Sperm		
Okada and Shimoizumi (1952)	*S. pulcherrimus*	Japan
Viguier (1900)	*Sphaerechinus granularis*	Algiers
Needham and Moore (1929)	*Dendraster excentricus*	Pacific Grove
Shapiro (1935)	*Arbacia punctulata*	Woods Hole
Reverberi (1947)	*Arbacia pustulosa* (5)	Naples
	Psammechinus microtuberculatus	Naples
No Test		
Chambers (personal communication)	*S. purpuratus* (2)	Berkeley
Gadd (1907)	*S. droebachiensis*	Murmansk
Rulon (personal communication)	*Dendraster excentricus*	Pacific Grove

Genus Incidence—Summary of Cases

Arbacia	15	*Dendraster*	3	*Psammechinus*	2
Paracentrotus	21	*Echinocardium*	4	*Sphaerechinus*	2
Strongylocentrotus	27	*Echinus*	1		

supply, in rare cases result in hermaphroditism. It is possible that other causative factors will have to be taken into account in any final analysis. Two of these are the incidence of hermaphroditism in the classification, and the distribution of the phenomenon geographically. On the first point, it is a striking fact that instances of hermaphroditism so far described are from relatively few genera and species, and these belong almost entirely to the order of the true sea urchins (Table I). Here we find five genera and ten species represented, while the sand dollars and

heart urchins furnish examples in one species each. One factor which may in part account for this preponderance of the true sea urchins is the accessibility and extensive use of these animals in scientific work. Most of them are littoral dwellers and are easily obtained, while members of the other two orders as a rule are dwellers of deeper waters and do not yield the quantity of eggs to be found in most of the sea urchins during the breeding season, and hence are little used. In view of the rarity of the phenomenon, the total number of individuals of a species examined is an important factor.

Despite the fact that ten or more species of sea urchins have been extensively used in experimental work, most of the cases of hermaphroditism have come from three genera, namely, *Arbacia, Paracentrotus* and *Strongylocentrotus.* It is notable that *Lytechinus,* which has been extensively used in southern stations, has not yielded a single case. Nor have examples of hermaphroditic sea urchins been reported from northern waters, except for the observation of a specimen of *S. droebachiensis* found fifty years ago at Murmansk by Gadd (1907). No cases have been reported from Scandinavia, none from Maine, none from the Oregon and Washington coasts. The distribution of hermaphroditic echinoids so far reported is in a band between N 35° and 55° in Europe, and between N 32° and 45° in North America.

More information and precise records with publication are needed to give a basis for assessing possible causative factors. It is important that those engaged in work with echinoid material examine animals opened for possible bisexuality, and where this condition is found, a sketch record be made of the positions of ovaries, testes, and ovotestes. Harvey's suggestion that cases of ovotestis may have been passed over as due to contamination when a worker has found occasional fertilized eggs among those freshly shed, is worth bearing in mind. Experiments to determine developmental potentialities both *inter se* and in outcrossing are of great interest and importance.

Summary

Two new cases of hermaphroditism in sea urchins are described. In a search for causative factors of bisexuality, a survey of incidence of the phenomenon in echinoids, both as to genera and geographical distribution, has been made. The suggestion of seasonal dimorphism is rejected.

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THE METABOLISM OF RADIONUCLIDES BY MARINE ORGANISMS. I. THE UPTAKE, ACCUMULATION, AND LOSS OF STRONTIUM ^{89} BY FISHES

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The revolution in biology which occurred about twenty years ago as a result of the utilization of isotopes has led to some problems which could hardly have been foreseen at the time. Biologists can no longer use radioactive material simply as a tool with which to solve specific problems; they are now forced to consider the effects of radioactivity which is introduced beyond their own control into an environment which they are studying. This becomes particularly important to marine and fresh water biologists who are interested in physiological or ecological problems which may be influenced in certain regions by an increase in radioactivity above the background which existed before the first atomic detonation at Alamagordo in 1945.

The weapons testing program of several nations, regardless of the type of blast, has increased the radioactivity of the seas. Underwater detonations, of course, contribute the largest percentage of their radioactivity directly to the water, but most of the fall-out from aerial bursts can be expected to appear ultimately in the ocean, since the land area of the earth is only about 30 per cent of the total. Run-off from the land will also increase the radioactivity of the seas. Far more important than the radioactivity which appears as a result of weapons testing, however, is the radioactivity which inevitably will be introduced into the oceans from nuclear power plant wastes and atomic-powered ships.

There are several important reasons for studying the metabolism of fission products and other radionuclides in marine organisms. First, several fission products are known to be potential hazards from a public health standpoint (N.B.S. Handbook 52). Second, almost nothing is known about the metabolism of these radioelements by marine species. Third, we cannot tell at present what potential ecological effects may be brought about through the deleterious action of radiation on the marine biota, but the possibility exists that some adverse changes, such as those which apparently occurred in White Oak Lake (Krumholz, 1956), might occur in estuaries and other inshore regions. It is therefore important to study these problems now, before the oceans become polluted with radioactivity, because the changes which may occur will be irreversible at least for several centuries.

The problems raised by the above considerations can best be solved by studying the metabolism of these radionuclides not only in individual organisms, but also in relation to the various trophic levels by way of the food chains. As desirable as such studies may be, it is impossible to undertake investigations of this magnitude

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for the entire marine biota. Thus, because of our special facilities at the Hawaii Marine Laboratory, we have confined our research to fishes which are representative of three distinct marine habitats, and include herbivores belonging to the second trophic level, and carnivores from the third and fourth trophic levels.

Strontium was selected for our initial studies for two reasons. First, it is chemically similar to calcium, and is therefore a "bone seeker." As such, if radioactive, it may interfere with the blood cell formation of many animals. Second, Sr^{90} has a half-life of about 28 years, so that the deposition of an atom of Sr^{90} into a tissue which has a slow rate of turnover may result in radiation exposure for the entire life of the animal. These characteristics make radiostrontium a particularly hazardous fission product. In these experiments we have used Sr^{89} because of its much shorter half-life ($\sim$ 53 days) which decreases the danger of contamination in the laboratory.

The particular objective of the present study was to measure the uptake, accumulation, and loss of radiostrontium by the various tissues and organs of selected species of fish when the isotope was given orally, by intramuscular injection, and by the immersion of the fish in sea water enriched with Sr^{89}.

Materials and Methods

The large pelagic fishes used in these experiments consisted of the black skipjack (*Euthynnus yaito*), the yellowfin tuna (*Neothunnus macropterus*), and the so-called dolphin (*Coryphaena hippurus*). These species are fast-swimming, wide-ranging carnivores which occupy the fourth trophic level. Among the small fish used, the papio (*Carangoides ajax*), and the aholehole (*Kuhlia sandvicensis*), are small carnivores common along the reefs and shores in the Hawaiian Islands and occupy the third trophic level primarily. The aholehole is also able to adapt itself to brackish water environments, and has even been found well into fresh water streams (Tester and Takata, 1953; Tester and Trefz, 1954). The third small species used, *Tilapia mossambica*, is a sluggish fish, predominantly herbivorous, but facultatively omnivorous, and may be placed in the second trophic level. It prefers brackish water, but is well adapted to either fresh water or sea water.

Carrier-free strontium^{89} was obtained from Oak Ridge and fed to the large fishes by filling gelatine capsules with cracker crumbs and a measured quantity of the isotope solution. The capsule was sewn into a small piece of fish muscle which was held just under the surface of the water by a weak thread. As the fish swallowed the bait, the thread was broken off. In this way the capsule could be given to a particular fish. In some instances small fishes were force-fed a gelatine capsule prepared in the same way. Others were fed by incorporating a measured amount of Sr^{89} into a gelatine solution which was allowed to solidify in a small plastic tube. The tube was put into the fish's stomach and the gelatine was extruded with the aid of a syringe. A dose solution was prepared by extruding the same quantity of radioactive gelatine into a volumetric flask.

The large fish were perfused with a mixture of two parts sea water and three parts distilled water. The brain, eyes, spinal cord and integument were removed, and the excised internal organs were further soaked in distilled water until no blood was apparent in the water. All the rinsings were added to the blood. The gut was opened, and any material remaining in it was flushed out. Only the eyes and

the visceral organs were removed from the small fish which were not perfused.

The remainder of the fish, consisting of muscle and skeleton for the large fish, or muscle, skeleton and integument for the small fish, was then put in a pressure cooker, brought to 20 pounds pressure and allowed to cool. After this treatment, the muscle was easily removed from the bones, and any flesh remaining on the gill arches was removed with warm formamide. Control experiments indicated that no leaching or loss of strontium occurred as a result of the pressure cooker treatment.

Wet weight of organs was obtained without blotting, and the tissues were dried at about 110° C. for 48 hours. The dried tissues were put in a muffle furnace which was brought to about 550° C. The furnace was then shut off and the samples left overnight. A slow stream of air was introduced in the oven to aid combustion.

The ash was ground and spread evenly on aluminum planchettes with the aid of water and a detergent, and dried under infra-red lamps. The samples were

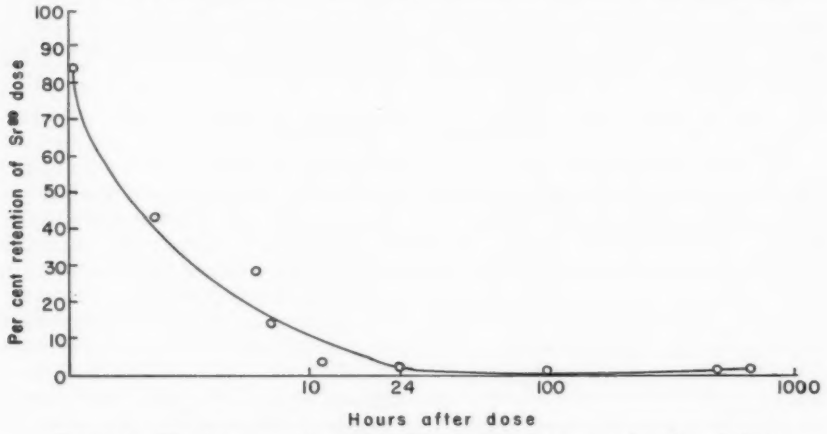

FIGURE 1. The decrease of ingested Sr^{89} in pelagic fishes as a function of time.

counted in triplicate when possible, using commercial counters and scalers. A minimum of 2560 counts was taken on each sample, including background. No corrections were made for back-scatter, self-scatter, or self-absorption. The latter is very small at the ash densities which were used (< 5 mg./cm.^{2}). The scalers were calibrated daily with Bureau of Standards nuclides, and the only correction applied was for radioactive decay. The counts/minute of the samples were compared with aliquots of the actual dose given in each instance. Specific details for each experiment will be described at the appropriate place.

RESULTS AND DISCUSSION

A. *Ingestion of Sr^{89} by large pelagic fishes*

Figure 1 shows that the excretion of a single dose is very rapid: about 50 per cent disappeared within a few hours, and only 1–2 per cent was left after 24 hours. This latter value persisted for the remainder of the experiment which lasted 27 days.

Table I shows the distribution of the Sr^{89} in the various organs and tissues of these fishes, and Figure 2 is a graph showing some of these data. This graph is presented as the percentage of radioactivity of the different organs and tissues in terms of the total radioactivity found in the entire fish when it was killed. It is

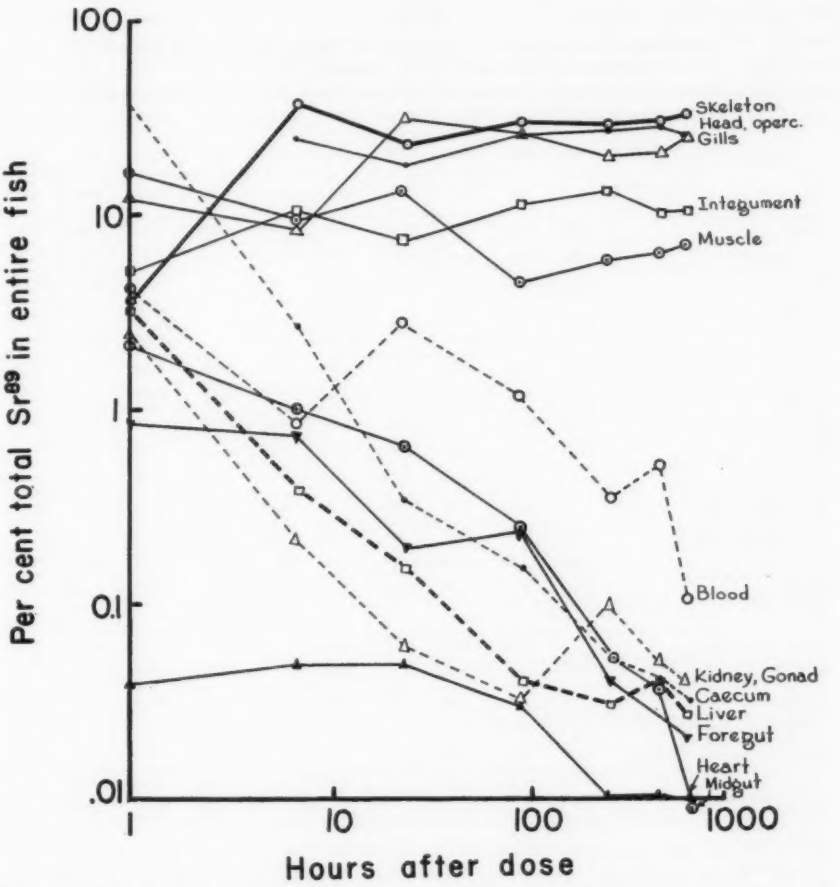

FIGURE 2. The distribution of a single ingestion of Sr^{89} in organs and tissues of pelagic fishes as a function of time.

apparent that the tissues are segregated into two groups with regard to strontium retention: the visceral, and the structural. The visceral organs and tissues, including the blood, kidney, foregut, midgut, hindgut, spleen, liver, caecum and heart, show a continuing decrease in radioactivity beginning one hour after the administration of the dose. The structural tissues, including the skeleton, head and

opercular bones, gill arches, integument and muscle appear to concentrate strontium rapidly to a level which is maintained more or less constant for a relatively long period of time. The turnover or excretion of strontium in these structures is therefore slow.

TABLE I

Accumulation of ingested Sr^{89} in the various organs and tissues of pelagic carnivorous fishes expressed as percentage of total activity

Tissue	Species	EV[1]	EV	EV	CH[2]	EV	EV	EV	NM[3]	EV	EV
	Dose in μc	5.55	480	240	51.0	240	464	464	464	371	371
	Duration in hours	1	2½	6	7	11½	24	96	264	480	648
Heart		0.04	0.01	0.03	0.049	0.11	0.05	0.028	0.01	0.014	0.007
Gall bladder		0.05	0.04	0.07	0.10	0.08	0.03	0.0001	0.01	0.004	0.002
Blood		4.21	6.68	15.00	0.85	8.07	2.73	1.14	0.35	0.51	0.12
Gill flesh		12.44	0.18	0.91	8.56	5.06	30.61	25.72	2.42	1.42	2.21
Gill bone			1.34	6.47		26.39			16.80	19.48	22.76
Caecum		37.01	7.67	7.84	2.70	2.64	0.34	0.15	0.05	0.04	0.029
Foregut		0.89	9.32	1.12	0.74	1.03	0.20	0.24	0.04	0.04	0.018
Midgut		2.28	14.16	16.50	1.08	1.48	0.65	0.25	0.05	0.036	0.003
Hindgut		11.78	3.98	21.26	2.26	0.11	0.15	0.024	0.03	0.015	0.016
Gut contents		—	48.32	12.73	0.056	19.65	—	0.10	0.013	—	0.0008
Head, operculum		—	0.41	1.09	24.99	6.28	18.33	24.58	28.18	29.91	24.58
Appendicular skeleton		3.60	0.40	1.19	36.21	8.45	23.69	30.32	29.15	30.47	31.43
Liver		3.34	1.48	3.04	0.39	2.46	0.15	0.04	0.03	0.04	0.027
Spleen		0.20	0.32	1.39	0.08	0.60	0.03	0.008	0.03	0.010	0.003
Tail		—	0.42	0.15	—	0.00	—	—	—	—	—
Brain, spinal cord		0.23	0.00	0.01	1.24	0.05	1.66	1.70	1.33	0.030	0.004
Eyes			0.04	0.06		0.60			2.02	1.34	1.34
Integument		5.28	1.69	0.86	10.20	5.89	7.69	11.37	13.73	10.25	10.51
Integument flesh (aliquot)		—	0.01	0.01	—	0.05	—	—	—	—	0.065
Integument scales (aliquot)		—	0.02	0.02	—	0.11	—	—	—	—	0.091
Gonad		2.40	0.09	0.47	0.22	0.08	—	0.004	0.03	0.023	0.020
Kidney			0.08	0.16		0.09	0.06	0.027	0.07	0.035	0.022
Light muscle		16.23	3.23	8.74	4.19	10.01	12.84	3.94	5.26	5.69	5.79
Dark muscle			0.10	0.86	5.25	0.70	0.72	0.48	0.47	0.63	0.95

[1] EV = *Euthynnus yaito.*
[2] CH = *Coryphaena hippurus.*
[3] NM = *Neothunnus macropterus.*

We do not interpret the departures from a smooth curve for any one organ to indicate a sequential pattern. In other words, a rise in the radioactivity of one organ and the fall of radioactivity in another are not necessarily linked by way of precursor relations. Each point on the graph represents a single fish, and individual differences can most likely account for the small deviations of the curves.

In order to study the sequential pattern of strontium metabolism, a much larger group of fish would have to be used, particularly since it is known that there is a very large difference in the time a food bolus remains in a fish as compared with another fish of the same species living in the same tank.

The rank order of radioactivity in the organ systems of these fishes is: skeleton, gills, integument, muscle and viscera. It is interesting to note that the dark muscle, which has a better supply of blood, has less radioactivity/gram ash than has the light muscle. Similarly, the "specific activity" of the gills, that is, the counts/minute/mg. ash, was considerably higher than that of the axial skeleton. Goldberg (personal communication) has analyzed yellowfin tuna for various metals, and found that the gill arches and filaments had considerably more strontium in them than had

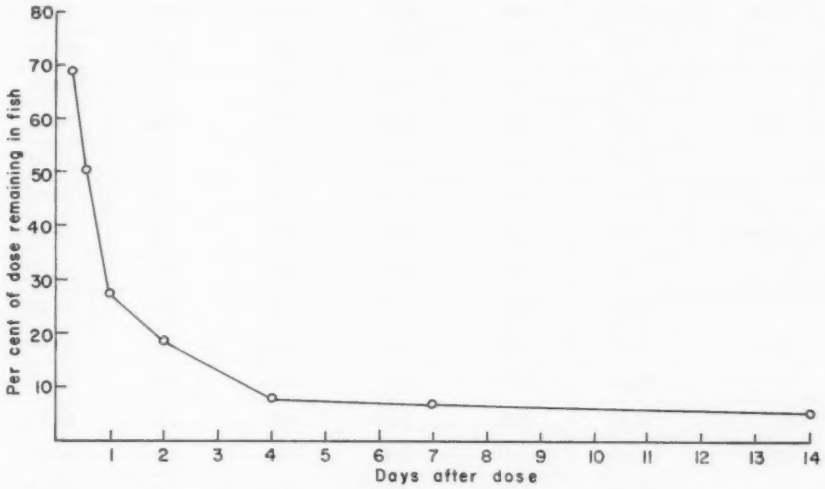

FIGURE 3. The decrease of ingested Sr^{89} in *Tilapia mossambica* as a function of time.

the bones. His values for strontium in the gills would therefore be a minimum value, since any flesh adhering to the sample would tend to dilute the strontium concentration. The main chemical difference between the gill arches and the remaining skeletal tissue is the presence of cartilage in the gill rakers. In our own work, and in the work of others (Jones and Copp, 1951), there is a suggestion that cartilage may have a greater capacity to exchange ionic calcium for strontium than has bone. For example, we have found a higher "specific activity" in the eye, which has cartilaginous ossicles, than we have found in skeletal bone. Moreover, Jones and Copp found that the uptake of strontium by the skeleton is more rapid in young rats than it is in adults. It is possible that the explanation of these differences might lie with an increased amount of Sr^{++} binding by the protein of the cartilage as compared to that bound by the protein of calcified bones. Perhaps differences in the amount of blood supplied to ossified and cartilaginous tissue, or some other properties of cartilage may also be involved.

TABLE II

Percentage of total radioactivity recovered in various organs of Tilapia mossambica. (Each horizontal column represents an individual fish given a dose of 20 μc)

Duration	Tissue					
	Integument	Eyes	Visceral organs	Gills	Muscle	Skeleton
2 hr.	10.58	0.26	31.88	28.02	9.44	19.81
	1.73	0.08	88.01	1.43	0.83	7.92
4 hr.	12.54	0.36	28.64	23.50	6.45	28.52
	0.32	0.07	65.81	11.34	10.88	11.58
	1.70	0.29	24.41	36.18	6.09	31.34
8 hr.	10.64	1.38	44.50	6.90	3.72	32.85
	8.01	0.24	44.40	15.83	5.46	26.06
	19.66	1.62	11.99	17.10	1.74	47.88
12 hr.	23.07	0.24	42.34	3.65	7.61	23.08
	4.63	1.26	78.36	1.95	1.61	12.19
	7.86	0.67	40.92	8.94	4.26	37.36
24 hr.	25.67	0.32	1.45	9.43	8.96	54.18
	24.93	0.36	1.60	8.60	3.52	60.99
	28.31	0.53	3.57	8.74	4.82	54.03
48 hr.	22.67	0.31	0.62	4.81	1.62	69.97
	5.79	0.83	3.32	14.35	7.39	68.32
	26.48	0.29	1.76	8.40	2.30	60.77
4 days	23.10	0.16	0.40	5.99	2.17	68.17
	26.71	0.27	0.64	8.08	2.38	61.92
	23.63	0.17	0.93	8.20	3.35	63.72
7 days	21.88	0.22	0.97	8.02	2.26	66.65
	20.84	0.21	0.57	8.28	1.92	68.18
14 days	31.42	0.24	0.17	8.92	1.97	56.73
	29.63	0.41	1.10	10.06	2.10	56.69
	20.39	0.22	0.51	8.07	1.24	69.57

B. *Ingestion of Sr^{89} by Tilapia*

Figure 3 shows that the rate of excretion of Sr^{89} by *Tilapia* is much slower than that by the pelagic fishes. About 50 per cent is still present after one day, and the time required to reach a more or less constant level is about four days. The amount

which persists is also larger than that observed with the pelagic fishes, although the variability among the *Tilapia* was fairly large. The *Tilapia* used in these experiments were fed the Sr^{89} in gelatine capsules containing cracker crumbs. Occasionally crumbs were observed in the carboys used to hold three of the experimental fish, and therefore the true dose could not be ascertained. The incorporation of the isotope in gelatine for the later experiments has apparently obviated this difficulty.

Table II presents the data concerning the percentage of the total radioactivity recovered in the various organs and tissues. Figure 4 is a graph of this information except that the ordinate is given in microcuries/gram fresh weight of fish.

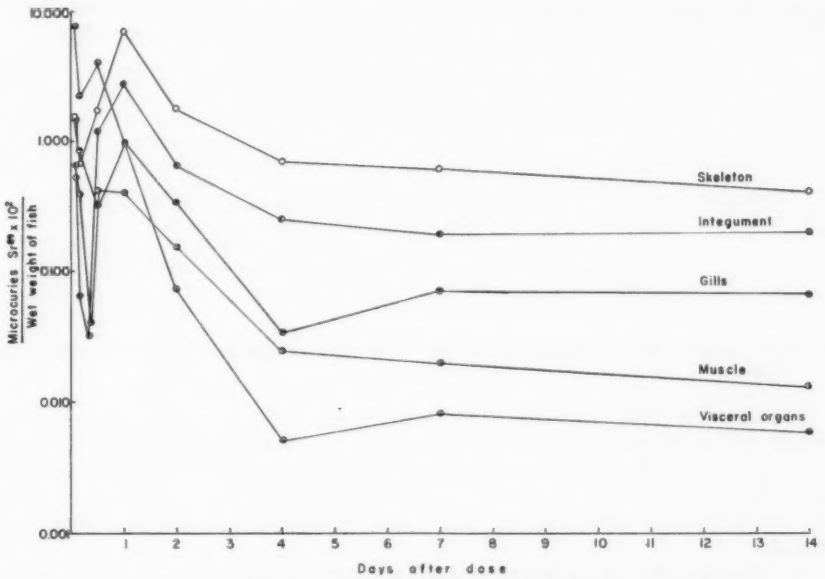

FIGURE 4. The distribution of a single ingestion of Sr^{89} in organs and tissues of *Tilapia mossambica* as a function of time.

The structural tissues account for the bulk of the radioactivity where approximately 90–95 per cent of the activity is present in both *Tilapia* and the pelagic fishes. Roughly, about 60 per cent is accounted for by the skeleton, 30 per cent by the integument, 10 per cent by the gills, 2 per cent by the muscle, and 1 per cent by the viscera. A larger percentage of radioactivity is found in the integument of *Tilapia* than in the integument of the other fishes, probably because a larger percentage of the body weight of this species is due to the large scales. The percentage of the total radioactivity in *Tilapia* was found to decrease in the following order: skeleton, integument, gills, muscle and viscera. The order in the pelagic fishes studied was skeleton, gills, integument, muscle and viscera.

C. *The ingestion of Sr^{89} by aholehole*

Five aholehole were each fed 48 μc of Sr^{89} in gelatine capsules and kept in running sea water 24 hours before killing. The entire fish was then dried and ashed, and Table III shows that the results are much more reproducible than they were using *Tilapia*. After 24 hours the latter fish retained approximately the same percentage of the dose as did the aholehole, but the range was between 2 and 20. No further experiments were conducted with aholehole at this time, but because of their apparent superiority as a laboratory animal we plan to use them in experiments which will be reported at a later date.

Although we have completed some experiments on the repetitive feeding of Sr^{89}, the uncertainty of the exact dose in some instances, and the excessive range of retention by *Tilapia* during short periods have caused us to omit these data here. The results of such experiments on other fish will be reported at a later date.

TABLE III

Percentage of Sr^{89} retained by aholehole 24 hours after ingestion

Fish	Per cent of dose
1	10.43
2	7.42
3	9.16
4	5.70
5	7.97
Aver.	8.13

D. *The uptake and accumulation of Sr^{89} injected intramuscularly into tuna and Tilapia*

Because our results showed that the biological half-life [3] of strontium in fish muscle fell in the same range as that found for the bones and integument, rather than with the soft tissues as was expected, additional experiments were devised to study the retention of Sr by muscle. These experiments also enabled us to study the metabolism of radiostrontium which was introduced by a method other than feeding.

A yellowfin tuna was injected with 288 μc of Sr^{89} at the base of the pectoral fin. The fish was killed and analyzed after it had been in running sea water for 19 hours. A comparison of the percentage of Sr^{89} recovered in the organs and tissues of a tuna which received the isotope by injection with that of a tuna receiving an oral dose (Table IV) shows that the digestive organs of the latter were relatively more radioactive than were the corresponding organs of the injected fish. This result is perhaps to be expected, but there are several other outstanding differences in the distribution of the isotope within this time period. The muscle tissue of the fish receiving the injection had almost three times the percentage of Sr^{89} as had the muscle of the fish receiving the radioisotope orally. The gills of the former, on the other hand, had only about half the percentage of Sr^{89} as had the orally dosed

[3] We define the term "biological half-life" in this paper to mean the time required for half the labelled strontium to be removed from the tissue or animal in question, exclusive of radioactive decay.

fish. The percentage of radioactivity in the integument and the skeleton of both fish was about the same, and it is not possible to state whether or not the differences found in the remaining organs are significant.

The fact that such a large percentage of the Sr^{89} was retained by the muscle suggested that it might be informative to study this process over a longer period of time. Thus, *Tilapia* were injected intramuscularly between the caudal and anal fin with Sr^{89} Cl_2 neutralized to about pH 6. The fish were kept in aerated, but not circulating, sea water for the first 24 hours, during which time samples of the water in the aquaria were removed. After 24 hours the fish were put in running sea water and removed at intervals. Figure 5 shows that the radiostrontium was rapidly excreted for about the first twelve hours, and that the rate decreased thereafter.

TABLE IV

A comparison of the percentage of Sr^{89} recovered in the organs and tissues of tuna related to the route of administration

Tissue	Oral[1]	Injected[2]
Integument	7.69	7.15
Gill bones	30.61	15.25
Gill flesh		1.81
Head, operculum	18.33	14.41
Appendicular skeleton	23.69	23.25
Light muscle	12.84	33.24
Dark muscle	0.72	1.51
Foregut	0.20	0.11
Midgut	0.65	0.36
Hindgut	0.15	0.07
Kidney/gonad	0.06	0.20
Heart	0.05	0.04
Caecum	0.34	0.16
Liver	0.15	0.13
Spleen	0.03	0.02
Gall bladder	0.03	0.37
Blood	2.73	1.79

[1] Skipjack, duration 24 hours.
[2] Yellowfin, duration 19 hours.

An analysis of the percentage of the dose retained by the fish confirmed the fact that after 24 hours comparatively little strontium was excreted, and that most of the strontium remaining was held by the fish for the duration of the experiment. These results are shown graphically in Figure 6. The points on the graph represent the average of three fish. The greater retention of the five-day fish as compared to the one-day fish can be ascribed to individual variation. The range of retention between the one- and the 21-day fish is of the same order of magnitude as the range of retention at any single time interval. In other words, the curve, neglecting individual differences, is very likely parallel to the abscissa.

The internal distribution of the injected Sr^{89} requires longer to reach a "levelling-off" than does Sr^{89} given orally (Fig. 7). In the former instance, the time required is about one week, whereas in the latter instance the "levelling" occurs within two days. In both situations, however, the percentage of the total radioactivity retained by each of the organ systems is the same.

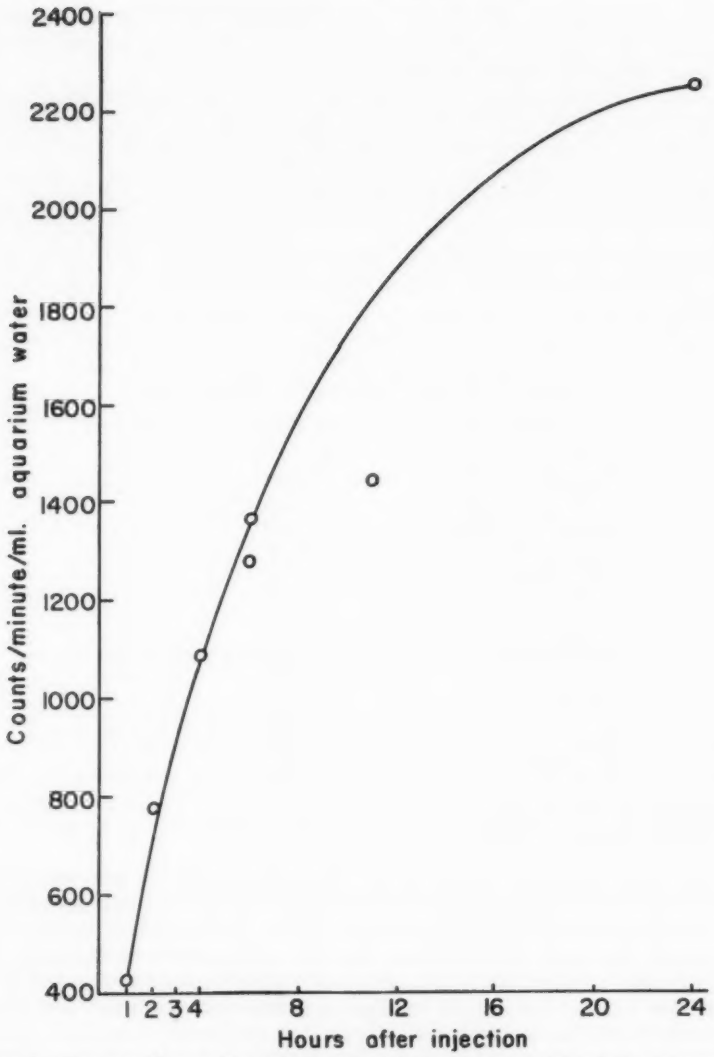

FIGURE 5. The rate of excretion of Sr^{89} injected intramuscularly into *Tilapia mossambica*.

The aquaria used in these experiments were inverted five-gallon carboys with the bottoms removed. Feces and other solid material thus settled to the neck of the carboy and could be removed through glass tubing which just penetrated the rubber stopper. In this way the feces were removed from the tanks six times during the first 24 hours at each sampling of the tank water. The average total

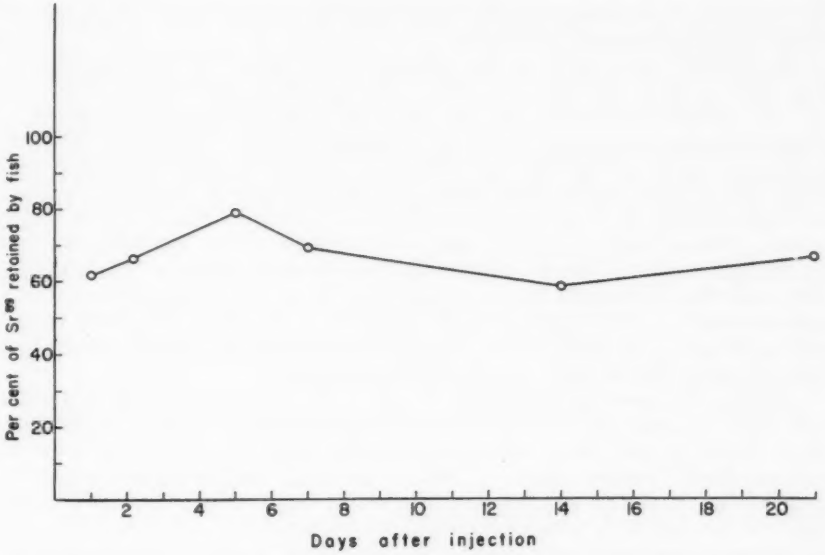

FIGURE 6. The continuing retention of Sr^{89} injected intramuscularly into *Tilapia mossambica*.

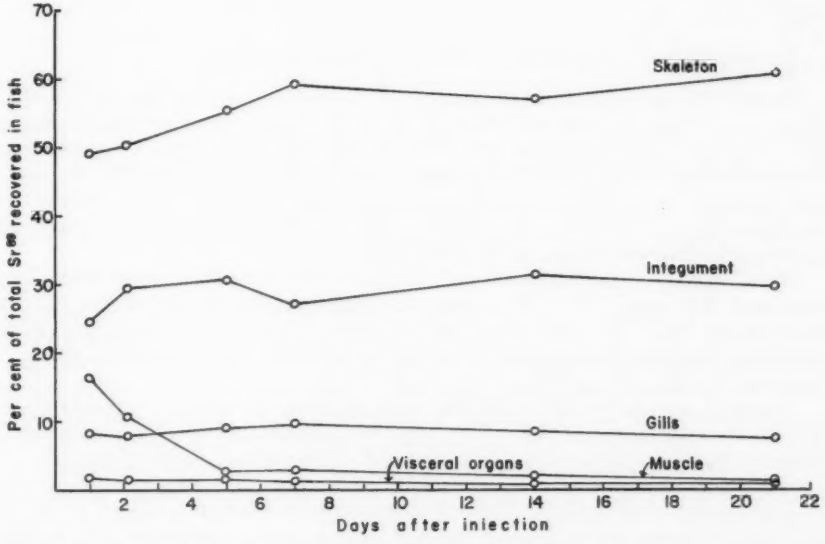

FIGURE 7. The internal distribution of Sr^{89} injected intramuscularly into *Tilapia mossambica*.

Sr^{89} recovered in the feces was 0.35 per cent of the dose at 24 hours, but the amount of leaching is unfortunately unknown.

The injected fish retained a much greater percentage of the dose than did the fish which received the strontium orally. These results suggest that the injected Sr^{89} was actually absorbed to a greater extent, and that the absorption of strontium through the gut is not efficient. Slow, continuous diffusion from the site of injection may also allow a larger amount to become incorporated into the various tissues. If the rate of blood supply to the muscles is less than that to the visceral organs, one might expect the muscle to retain the strontium for a comparatively longer period. The fact that the dark muscle of tuna retained much less strontium than did the light muscle (Table I), and the fact that the dark muscle is better supplied with blood than is the light muscle, suggest that the degree of vascularization is of some importance. One might therefore reasonably expect the cartilaginous tissues, such as the gill rakers and gill rays, to retain the strontium for longer periods than does the bone which is better supplied with capillaries.

Although the amount of strontium in tuna muscles and visceral organs is of the same order of magnitude (Goldberg, personal communication), some specific binding of strontium may occur with muscle protein which does not occur with the proteins of the visceral organs. Further, the very long biological half-life of strontium in the muscle suggests that fish muscle may not be in such a "dynamic state" as one ordinarily assumes according to the researches of Schoenheimer and his associates (Schoenheimer, 1942). Moreover, there is evidence that mammalian muscle protein has a very much slower turnover rate than has the proteins of the visceral organs (Tarver and Schmidt, 1942). Therefore, the slow turnover of strontium in the fish muscle may be only a reflection of the slow turnover of muscle in general.

E. *The uptake and accumulation of Sr^{89} in solution by Tilapia*

Because the pattern of distribution of Sr^{89} in the tissues and organs of several species of fish appears to be similar, both when the isotope was given orally and by injection, one might extrapolate and conclude that regardless of the mode of entry, the internal distribution of Sr^{89} ultimately would be the same. However, to secure more information on this point, and to ascertain whether or not fish could take up strontium directly from the sea water, a situation which is possible in nature, the experiments described below were carried out.

Six *Tilapia* were put into each of four tanks containing 20 liters of filtered sea water and 1744 μc of Sr^{89}. The water was aerated during the experiment, but the fish were not fed. The total amount of Sr^{89} available during the experiment can be considered constant, since even at 21 days, the total Sr^{89} removed by six fish in a tank was less than one per cent of the available dose.

Figure 8 shows the rate of uptake from solution in terms of microcuries of Sr^{89}/gram fresh weight of fish. The rate slows down considerably after about a week, but uptake is apparently still continuing. The ordinate on the right indicates that within 21 days, the ratio of internal Sr^{89} to external Sr^{89} is still less than one.

The internal distribution of the Sr^{89} taken up from solution is shown in Figure 9. The Sr^{89} found in the skeleton is about 40 per cent as compared with a value close to 60 per cent when the strontium is fed or injected. The amount found in

the integument in all cases is about the same, and the gills and muscles show little variation. The amount found in the visceral organs, however, is markedly different. The individual organs were not ashed and counted separately because of their very small size, so it is not possible to state in what organ or organs the very large percentage of Sr^{89} was located.

Since marine fish in general swallow more water than do fresh water fish to maintain proper osmotic balance, it is possible that the principal route of entry of the isotope in solution is by way of the gut. However, our direct feeding experi-

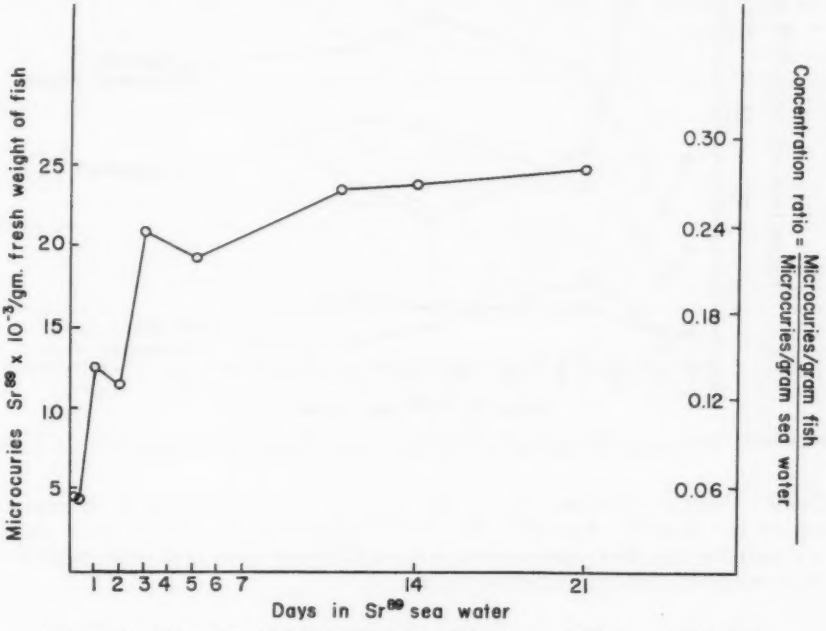

FIGURE 8. The uptake of Sr^{89} in solution by *Tilapia mossambica*, expressed as the concentration ratio.

ments with a variety of fishes indicate that strontium is rapidly eliminated from all the visceral organs. How is it, then, that so much strontium remains in the visceral organs when the fish is immersed in the isotope? A probable explanation is that the fish is being fed the isotope continually, in effect, every time it swallows. Since the concentration of Sr^{89} was found to be higher in the sea water than in the fish, only a small amount of sea water present in the gut would account for the large percentage of total Sr^{89} which was found in the visceral organs. The concentration of Sr^{89} in the sea water was 8.7×10^{-2} μc/ml. Assuming arbitrarily that 50 per cent of the total Sr^{89} of the fish was in the visceral organs (Fig. 9), this amount equals about 12×10^{-3} μc/gram fresh weight of the organs. If only from 0.1–0.2 ml. of sea water was present in the gut, this would account for the radioactivity

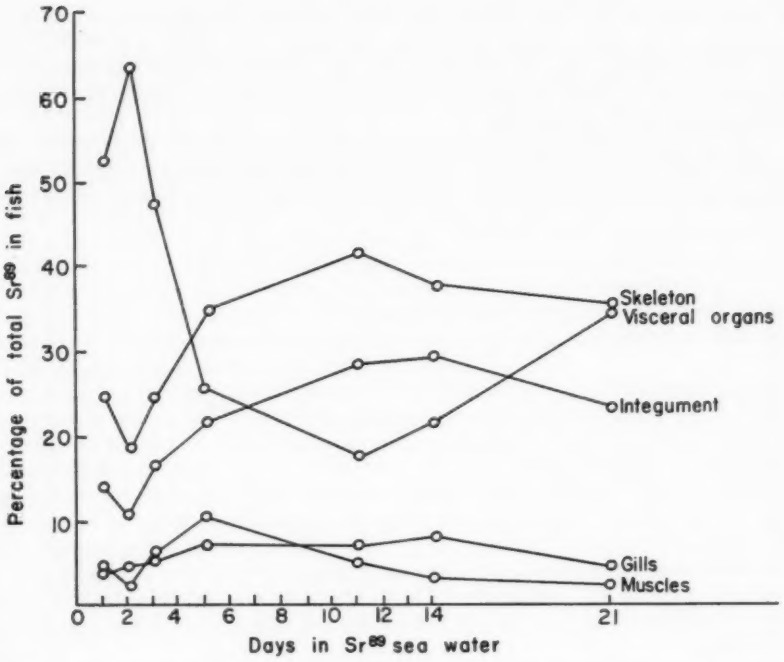

FIGURE 9. The internal distribution of Sr^{89} taken up by *Tilapia mossambica* from sea water.

found. What is important, then, is the rank order of radioactivity in the various organs and systems. Excluding the visceral organs for the reasons given above, it is seen that the other organ systems studied fall in the same rank order regardless of the mode of entry of the radiostrontium.

SUMMARY

1. The ingestion of Sr^{89} by large pelagic fishes results in the excretion of most of the isotope in a few hours. The small percentage remaining after one day persisted for the 27 days of the experiment. The strontium is rapidly eliminated from the visceral organs and tissues, but the structural tissues, including the bones, gills, integument and muscle, maintain their strontium level more or less constant. The turnover of strontium in these latter tissues is therefore slow.

2. Dark muscle, which has a better blood supply than light muscle, retains less Sr^{89}. Similarly, bone, which is better supplied with blood than is cartilage, retains less Sr^{89} than the gill arches or the cartilaginous eye ossicles.

3. The excretion of Sr^{89} by *Tilapia mossambica* is much slower than it is by the pelagic fishes. The percentage of the dose retained is somewhat larger, and most of the radioactivity is found in the structural tissues.

4. About three times as much Sr^{89} was found in the muscle of an injected tuna as compared with another fish receiving the isotope orally. The gills of the former fish had only about half the activity found in the latter. From 60–70 per cent of the dose injected into *Tilapia* muscle was retained by these fish for 14 days. The long biological half-life of Sr^{89} in fish muscle is contributing evidence for the slow turnover of muscle tissue in comparison with such tissues as liver or kidney.

5. *Tilapia* were able to concentrate Sr^{89} directly from the sea water, although the ratio of Sr^{89} in the fish to the Sr^{89} in an equal weight of sea water was only about 0.3 after three weeks. Except for the visceral organs, the rank order of the retention of radioactivity in the various tissues is skeleton, integument, gills and muscle. This is the same distribution as was observed after oral administration of Sr^{89}. Because marine fish swallow water continually, a small amount of water in the gut might account for the relatively large percentage of radioactivity found in the viscera.

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THE METABOLISM OF RADIONUCLIDES BY MARINE ORGANISMS. II. THE UPTAKE, ACCUMULATION, AND LOSS OF YTTRIUM^{91} BY MARINE FISH, AND THE IMPORTANCE OF SHORT-LIVED RADIONUCLIDES IN THE SEA [1, 2]

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A study of the metabolism of radioyttrium is important for two reasons: first, yttrium^{90} occurs as the daughter of long-lived strontium^{90}, an element which has considerable interest from the standpoint of public health, and second, yttrium^{91} occurs as a direct fission product to the extent of about four per cent of the total radioactivity present in a fission product mixture one year old. Fission products are being introduced into the seas to a small extent as a result of fall-out, and also from nuclear reactor plants located near the seaboard. The latter situation now occurs in the Irish Sea near Harwell, and more activity of this type may occur as reactor plants increase in number. Spooner (1949), in a noteworthy study on the metabolism of radioyttrium by marine algae, showed that this element was capable of being concentrated by certain algae, and thus stimulated the interest of marine biologists to learn more about the metabolism of the nuclide of mass^{90}.

Because we had begun experiments on the metabolism of strontium^{89} and strontium^{90}, both of which contain a certain amount of yttrium^{90}, we were interested in comparing the metabolism of these two elements in marine fish. Although the ratio of Sr^{90}: Y^{90} in an equilibrium mixture is about 3000: 1, it was conceivable that some of the results we obtained might have come from the small amount of yttrium present.

In a number of instances the concentration of certain elements is sometimes greater within a marine organism than it is in the surrounding sea water (Noddack and Noddack, 1939; Vinogradov, 1953). For example, one cannot predict, let alone explain, why one species of green algae will accumulate yttrium almost exclusively from a mixture of strontium and yttrium, while another accumulates strontium exclusively. Thus, Rice (1956) reports that *Carteria* sp. takes up 100 per cent of its radioactivity from the strontium in a Sr^{90}–Y^{90} mixture, but *Chlorella* sp., which is also a member of the Chlorophyceae, has 95 per cent of its radioactivity in the form of yttrium. Such tremendous differences in accumulation cannot be explained on the basis of size, that is, surface per gram protoplasm. Rather, the explanation is more likely to be one involving the chemical nature of the surfaces among the different algae. Because closely related algae are able to concentrate one element more than another, it is not unreasonable to suspect that one organ of a fish might have a greater avidity for yttrium than it has for strontium. Unfor-

[1] This work was carried out with the aid of Contract No. AT(04-3)-56 between the Atomic Energy Commission and the University of Hawaii.

[2] Contribution No. 83, Hawaii Marine Laboratory, University of Hawaii.

tunately, the small size of the fish used in these experiments did not permit us to separate the visceral organs in detail, so that we can only report the amount of radioactivity found in the integument, skeleton, gills, muscle, and in the combined visceral organs.

Materials and Methods

Yttrium^{91}, obtained from Oak Ridge, was incorporated into a two per cent gelatine solution, and 0.5 ml. was drawn up into a piece of Tygon tubing of small diameter. When the solution solidified, it was extruded directly into the fish's stomach with the aid of a syringe. The dose was 5.5 microcuries. The fish used were *Tilapia mossambica,* each of which weighed about 100 grams. After the radioyttrium was administered, three fish were put into a single carboy with 20 liters of sea water which had been filtered through a No. 4 Mandler filter. Four carboys were used for the four time intervals of 1, 2, 4 and 14 days. The water was aerated, but the fish were not fed. Twenty-four hours after the administration of the yttrium, the fish were put in tanks with running sea water where they were kept until killed rapidly by flooding the gill chamber with ether. After removing the eyes and the visceral organs, the remainder of the fish was put in a pressure cooker which was brought to 20 pounds pressure and then allowed to cool. This process allows the skeleton and integument to be separated easily from the muscle with no leaching of the radioisotope. The separated organs and tissues were dried and ashed at about 550° C. The ash was spread on aluminum planchettes with the aid of a wetting agent, dried under infra-red lamps, and counted with a G-M tube and a conventional scaler for a minimum of 2560 counts. Yttrium^{91} has a maximum energy of 1.5 Mev. No corrections were made for the self-absorption which was very small at the densities employed (< 6 mg./cm.^{2}). An aliquot of the dose was counted for reference and to correct for decay.

Results

Figure 1 shows that radioyttrium is very rapidly excreted by *Tilapia.* After two days, the fishes retained only about two per cent of the ingested dose (1.6 ± 0.5). In a similar experiment, the average amount of Sr^{89} retained by *Tilapia* after two days was 20 per cent, and even after 14 days, the average retention was about six per cent (Boroughs, Townsley and Hiatt, 1956). The actual absorbed dose may be something less than these values, because marine fishes swallow water in order to maintain their osmotic balance. A small amount of recently swallowed water would therefore be trapped in the gut unabsorbed, but contributing to the radioactivity in the visceral organs. Any feces remaining in the gut would also add to the radioactivity of the visceral organs. In both instances, however, the amount would be small.

Figure 2 indicates that most of the yttrium is retained in the viscera. In rats, the liver, kidney and spleen accumulate yttrium (Hamilton, 1948), but our methods did not permit us to localize the accumulation of yttrium in the visceral organs. Future experiments to disclose this are underway. Assuming that all the radioactivity recovered in the 14-day fishes was absorbed (1.3 per cent of the dose), the

viscera retained 43 per cent, the muscle 29 per cent, the skeleton 16 per cent, the integument 8 per cent, and the gills 4 per cent. The large percentage of yttrium accumulated in the fish muscle was wholly unexpected, for in rats 65 per cent of the absorbed dose was found in the bones (Hamilton, 1948).

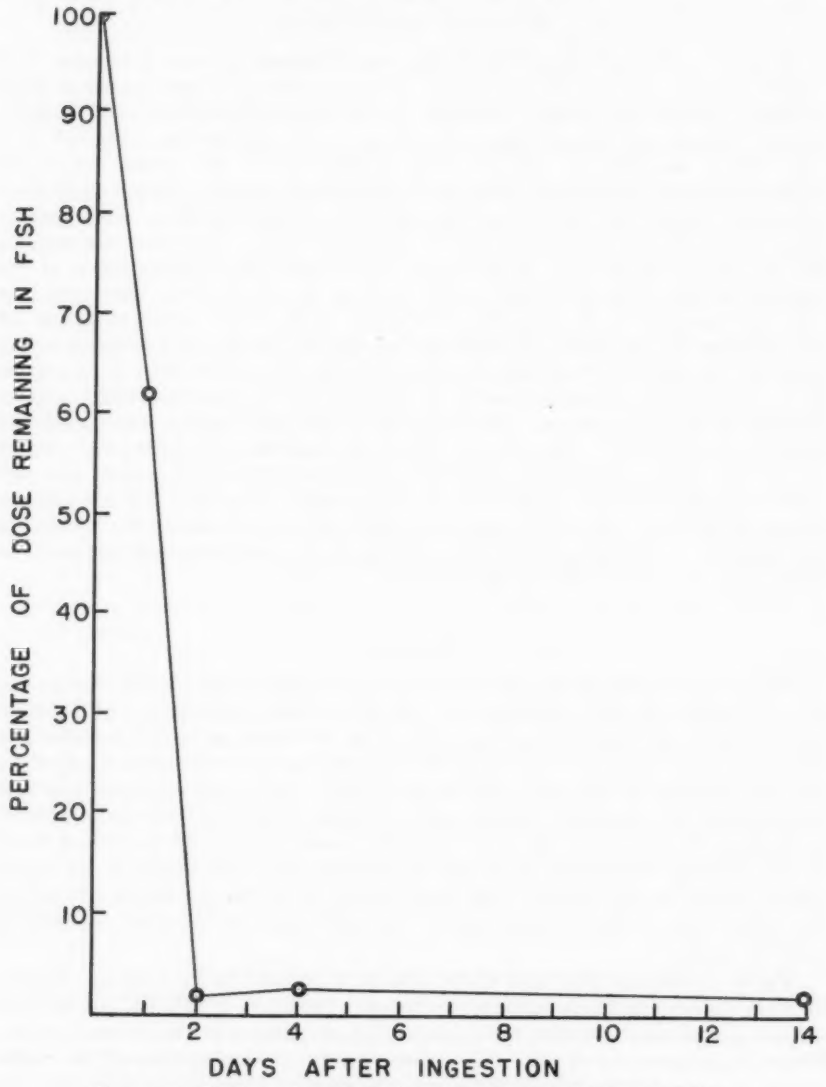

FIGURE 1. The loss of yttrium^{91} after ingestion by *Tilapia mossambica*.

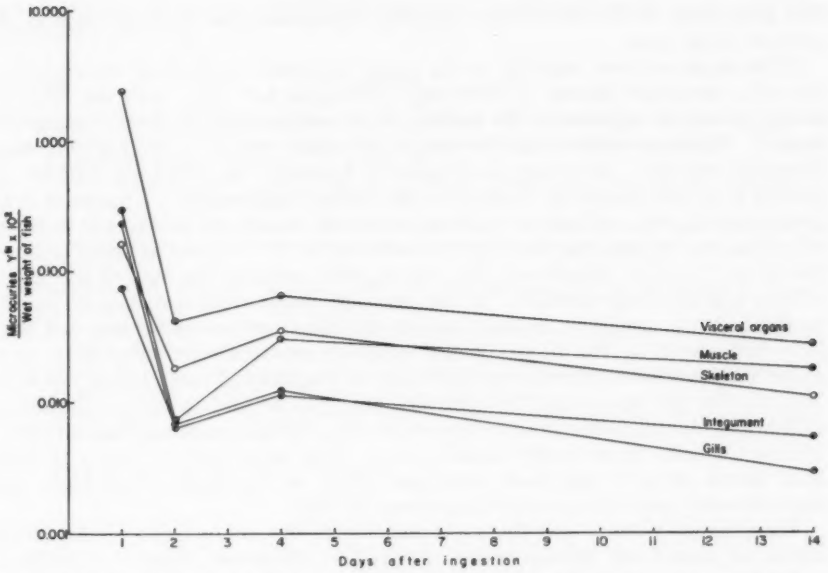

FIGURE 2. The internal distribution of yttrium[91] fed to *Tilapia mossambica* via stomach tube.

DISCUSSION

Similar experiments with radiostrontium (Boroughs, Townsley and Hiatt, 1956) have indicated that in 14 days, *Tilapia* have about 60 per cent of the absorbed dose in the skeleton, 28 per cent in the integument, 9 per cent in the gills, 2 per cent in the muscle, and 1 per cent in the viscera. The rapid excretion of radioyttrium by *Tilapia,* coupled with the different patterns of internal distribution, indicate that the metabolism of strontium and yttrium in this species is markedly different. However, this is not too surprising in view of the difference in chemical behavior between these two elements. Studies on the accumulation of strontium by pelagic fish (Boroughs, Townsley and Hiatt, 1956) showed that this element is rapidly absorbed by all the tissues, but is also rapidly lost from the visceral organs and the blood. Similar experiments with *Tilapia,* a small sluggish fish, were unsatisfactory because of the large variability in the amount of Sr^{89} absorbed over periods up to 24 hours. We therefore made no attempt to follow the pathway of yttrium immediately after ingestion, but very likely yttrium is not actually absorbed to the extent that is strontium. At 24 hours, about 60 per cent of the dose was still in the entire fish, but most of this was in the gut. However, at least 5 per cent of the dose was absorbed by the muscle alone. This is at least 100 times more than the amount absorbed by rats from an oral dose (Hamilton, 1948), and, moreover, represents a minimum value for *Tilapia.*

The maximum percentage of ingested strontium which was recovered in the feces of *Tilapia* during 24 hours was less than 1 per cent. The feces were removed six or seven times during this interval, but some leaching of Sr^{89} may have occurred.

The percentage of Y^{91} recovered in parallel experiments has been as much as 20 per cent of the dose.

If the length of time required for an organ to excrete one-half of its concentration of a particular element is very long (biological half-life), then the effective biological half-life approaches the half-life of the radioisotope involved (radioactive decay). Elements which lodge in mammalian bones appear to have a very long biological half-life. According to Figure 2, however, the biological half-life of yttrium in all the tissues of *Tilapia* is of the order of one month. This value is of course only a guess, and long term experiments will have to be carried out to verify this estimate. In man, the body burden tolerance for Y^{91} is given as about 15 times that of Sr^{90} (N.B.S. Handbook 52), and in both instances the bulk of the radioactivity appears in the skeleton. In one year, the radioactivity owing to Y^{91} would be reduced to a negligible amount because of both excretion and decay, but very little Sr^{90} would be lost in this time. However, in organisms other than man, particularly marine organisms, one year may be a substantial part of their life span, and it is for this reason that we urge that attention be paid to the metabolism of radioactive fission products other than strontium. While strontium may constitute the most serious *direct* health hazard to man, long term effects of other shorter-lived fission products may have significant effects on the shorter-lived biota, and thus ultimately may also prove of importance to man.

There is no evidence that the slight increase in the radioactivity of the oceans has as yet caused any adverse ecological changes. Moreover, there is no evidence that adverse ecological changes will occur even as a result of the introduction of much larger amounts of radioactivity from nuclear reactor plants which are certain to be established within the next decade or so. If the present power requirements of the world are to be met with the aid of atomic energy, it is likely that a sort of steady-state condition will occur with regard to the added radioactivity in the oceans—the result of a balance between the rate of introduction of radioactive wastes, the rate of physical decay, and the rate of biological turnover. It is therefore imperative that marine biologists study in great detail the problems of the uptake and accumulation of fission products, the transfer of these nuclides back and forth among the trophic levels, and the direct, long-term effects of the nuclides in specific regions. Estuaries and the littoral zone will most likely have a higher concentration of radioactivity than the open sea, and it is in these regions that the bulk of the world's marine resources is produced.

Summary

Only about 2 per cent of an ingested dose of yttrium^{91} was left in *Tilapia mossambica* after two days. This is much less than the amount of strontium retained by *Tilapia* in similar experiments. About 40 per cent of the radioisotope remaining is found in the visceral organs, but the muscles retain about 30 per cent after 14 days. The skeleton retained less than 20 per cent, the integument about 10 per cent, and the gills 5 per cent. These findings are in marked contrast with those obtained with strontium^{89} in similar experiments. Attention is focused on the fact that yttrium^{91} may have little direct effect on man compared with the possible effects of Sr^{90}, but the retention of this and other short-lived fission products in

marine organisms having a brief life span may possibly affect the biota, and thus affect man indirectly.

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IMMOBILIZING AND PRECIPITATING ANTIGENS OF PARAMECIUM

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The pattern of inheritance of the immobilization antigens of *Paramecium aurelia* and the serological basis for the specific immobilization reactions have been studied by Sonneborn (1951), Beale (1952), van Wagtendonk and van Tijn (1953) and Finger (1955). The systems of transformation from one serotype to another have also been investigated (Sonneborn, 1950a; Beale, 1948) and possible mechanisms suggested (Kimball, 1947; Sonneborn, 1950b; Delbruck, 1949). Further work on the unique systems of inheritance and stability exhibited by the immobilization antigens has been hampered by the lack of a rapid, reproducible method for assaying them. We have now been able to detect these antigens through the development of a method which involves a modification of the techniques of Oudin (1952) and Oakley and Fulthorpe (1953). Specifically, a particular band formed by the diffusion of two reactants (antigen solution and antiserum) from opposite ends of an agar column has been identified as a complex, in part, of precipitated immobilization antigen and its homologous antibody. The present paper deals with the immobilization antigens and this *in vitro* method for detecting them.

Materials and Methods

Sera against whole animals prepared according to the methods described by Sonneborn (1950c) against several stocks of varieties *2*, 4 and 8 were employed. These were tested with variety *2* antigen extracts of stock 3 and extracts of animals that were derived from the variety *2* stocks 7, 30 and 35. The survey of antigenic types found in variety *2*, upon which much of the work reported here is based, has been submitted for publication. The antigen solutions were prepared in several ways: (1) Cilia were obtained from paramecia and extracted as follows (Preer and Finger, unpublished). Six million animals were concentrated by centrifugation and one volume placed in twenty volumes of 0.22% sodium chloride buffered at pH 7.8 with 0.01 *M* sodium phosphate. At the end of ten minutes the animals were centrifuged lightly at 890 *g* for two minutes; the supernatant (containing mainly cilia) was retained and again centrifuged lightly. Any sediment of animals and trichocysts formed was then removed and the process repeated until the supernatant was free of animals. The supernatant was then centrifuged for two minutes at 24,000 *g* in order to sediment the cilia. Then one ml. of 0.9% sodium chloride, buffered to pH 7.0 with 0.01 *M* sodium phosphate, was added to the centrifugate. After 24

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hours at room temperature this suspension was centrifuged at 24,000 *g* for two minutes and the clear supernatant used as antigen. (2) Lyophilized animals were placed in a 0.9% sodium chloride solution overnight in a refrigerator, centrifuged at 24,000 *g* for two minutes and the supernatant used as antigen. (3) Animals were concentrated by centrifugation, placed in a deep freeze, and, after thawing at room temperature, the entire brei was used as antigen. (4) A brei was made by repeatedly forcing living animals from a syringe against the walls of a glass cylinder at room temperature. The resulting homogenate was then centrifuged for ten minutes at 24,000 *g* and the supernatant used as antigen. These four preparations were not equally effective in yielding the immobilization antigen and are listed in increasing order of efficiency. When quantitatively comparable data were desired the antigen solutions used were, of course, obtained by the same method.

The method of gel diffusion has been given in detail by Preer (1956). It is as follows: Pyrex tubes, approximately 2-mm. inside diameter, were coated with a 0.1% agar solution, evacuated, cut into 4-cm. lengths and flame-sealed at one end. To set up a double-diffusion test 0.01 ml. of antiserum was added with a syringe to a tube held upright in a Cartesian diver loader. Then 0.6% washed, merthiolated, and buffered (pH 7.0) agar maintained at 60° C. was carefully layered with a warm syringe until an agar column of 6–8 mm. height was reached. A third layer of 0.01 ml. antigen solution was placed on the agar after it had solidified. The tube was then sealed with Picene cement and placed in a horizontal position at 24° C. When serum dilutions were used, normal serum was used as the diluent. This was to prevent mixing of antiserum with the warm agar at the time of layering. The diluent for the antigen was usually 0.9% sodium chloride or, less frequently, 10% Ringer's solution.

The location of a zone of precipitation within the agar was used as a measure of the concentration of one of the reactants (antigen or antibody) contributing to the band when the concentration of the other reactant was kept constant (Preer, 1956). The position of a band was measured after 24–72 hours with a binocular dissecting microscope provided with an eyepiece micrometer.

Variations on these general techniques, *e.g.*, mutual dilution, absorption experiments, etc. are described below.

Results

When a solution of several antigens is diffused against homologous antiserum, bands of precipitate will be formed as a result of the specific complexing of antigens with antibody. Because the position of a band is dependent on several factors (concentration of reactants, diffusion coefficients, etc.), each serologically distinct precipitating system usually appears as a separate zone of precipitation. Occasionally, however, two or more precipitating systems may, through a fortuitous combination of these factors, appear as a single band. Therefore the number of bands will represent the minimum number of antigen-antibody systems present.

Many workers have demonstrated that *P. aurelia* may manifest a number of different antigenic types, called "serotypes," and designated by the letters A, B, C, etc. When animals of a given serotype are placed into a suitable dilution of antiserum prepared against that type, their locomotion slows, and they become immobilized. Each serotype is serologically distinct from any of the others, an animal

of a particular serotype generally becoming immobilized only when placed in homologous antiserum. If a number of antisera are allowed to diffuse against extracts of homologous serotypes and against extracts of heterologous serotypes, then it should be possible to correlate the presence of certain bands with these specific immobilization antigen-antibody systems.

When animals of the G serotype were used as the source of antigens and G antiserum diffused against the antigen solution, a band formed that was not present when an antigen solution prepared from non-G animals reacted with a G antiserum. Six different stocks of *P. aurelia*, all of serotype G, possessed a similar antigen, an antigen absent in extracts of two stocks of a serotype other than G and in non-G animals of two other species, *P. caudatum* and *P. polycaryum*.

Analogous results were obtained with C antigen-antiserum systems. A band appeared in these homologous systems that was missing when the antigen solution was obtained from non-C animals.

To provide further evidence that this antigen was found only in homologous serotypes, absorption experiments were performed. It was found that extracts of several stocks manifesting the G serotype were able to remove the specific precipitin from G antisera while several stocks of a different serotype and non-G *P. caudatum* and *P. polycaryum* preparations were ineffective.

The precipitating antigen associated with a particular serotype was further homologized between stocks through the use of a mutual dilution technique (Oudin, 1952; Telfer and Williams, 1953). Although all antigen preparations were able to form a distinctive band when diffused against homologous antisera, it was possible that the bands formed with different antigen preparations did not represent the same antigen-antibody systems, the correspondence being coincidental. An antigen forming a band against a particular serum may be identified with an antigen from a second extract by mutually diluting the two extracts, one with the other, and noting whether the bands formed behave independently, as though diluted with a neutral reagent, or act to reinforce each other. In order to employ the mutual dilution technique with maximum effectiveness, the concentrations of serum and antigen solution were chosen so as to eliminate most or all of the bands in the system, aside from the ones being compared, and still leave a band intense enough to withstand a two-fold dilution of antigen. For optimum resolution in a mutual dilution series the concentrations were adjusted so that the bands in the two preparations being compared formed at widely separated positions in the original undiluted systems. As a result of the mutual dilution studies, the bands formed with different preparations of the same serotype were shown to represent the same antigen-antibody system.

In summary, then, a study of serotypes G and C has shown that animals of each serotype have a specific precipitating antigen which is lacking in animals of other serotypes. It seems likely, then, that these antigens are the immobilizing antigens. Such an immobilization antigen can be identified in gel-diffusion systems in several ways: (1) a comparison of bands formed with homologous and heterologous antigens; (2) specific absorption by heterologous extracts of all antibody from a homologous antiserum, but the one that forms a zone of precipitation with homologous extracts; and (3) the mutual dilution of an extract with an antigen solution which forms several bands, one of which is known to be formed by the specific precipitinogen.

Although the immobilization antigen and the specific precipitating antigen are probably the same, there is evidence that the precipitated band may not be composed solely of immobilizing antigen and immobilizing antibody. The evidence has been obtained from a comparison of immobilization titers of antisera and precipitin titers as measured by the specific band position.

This specific band was identified as above by diffusing antisera against antigens extracted from two clones with the same genotype but of contrasting serotypes. The eleven sera titered for antibody forming this band were prepared against several different stocks and were known to immobilize animals of the G serotype in concentrations of 1 : 12.5 or less after two hours at room temperature. Eight of these anti-G sera, all having an immobilization titer of 1 : 50 or greater, precipitated

TABLE I

Comparison of immobilization titers and gel diffusion band titers of antisera against P. aurelia

Serum	Anti-G titer	G-precipitin titer	Anti-C titer	C-precipitin titer	Serum	Anti-G titer	G-precipitin titer	Anti-C titer	C-precipitin titer
P#23	100	40	0	0	F#10	0	0	100	100
P#16	100	9	0	0	P#8	0	0	100	65
P#2	75	100	0	0	F#17	0	0	50	30
P#12	75	100	0	0	F#11	0	0	40	90
P#7	50	15	0	0	F#4	0	0	25	25
P#14	50	30	0	0	F#5	0	0	25	45
P#17	50	15	0	0	F#12	0	0	20	9
F#15	20	45	15	4	P#18	0	0	15	4
F#18	15	3	0	0	F#3	0	0	15	7
F#14B	5	5	20	11	P#19	0	0	5	7
F#13B	3	1	0	1	F#1	0	0	3	0
					P#4	0	2	0	0
					P#21	0	3	0	0

The immobilization antibody titers (anti-G and anti-C titers) and the gel diffusion precipitin titers are presented on a scale with sera having the greatest concentration of antibody listed as 100, and the antibody concentration of all other sera, as compared with these sera, being denoted by numbers from 0–100. In this way, a serum with half the antibody content of the strongest sera would be given the number 50, etc. The titers presented above represent the means of several series of titrations.

a single band against the G antigen solution that was absent when preparations of the C serotype were used as reactants. Of the three anti-G sera that showed the same number and type of bands with both G and C antigen solutions, two had immobilization titers against both serotypes (F#15 and F#14B) and one (F#13B) had negligible anti-C precipitin titer. These data are presented in Table I where the immobilization titers of 24 antisera against the G and C serotypes are compared with the precipitating antibody concentration as determined by band position.

Similar results were obtained with anti-C sera when the sera were titered against homologous and heterologous solutions. Ten of thirteen antisera gave a band with C antigen solutions not present when G antigen solutions were used. Of the three homologous antisera that did not precipitate this band, one had a very low immobilization titer (F#1), and two also had anti-G immobilization titer. Thus, any differences that may have existed due to the C immobilizing antibodies

would be obscured (F#15 and F#14B). Seventeen sera prepared against several serotypes (omitted from Table I) and with neither G nor C immobilization antibody presented essentially identical band series with G and C antigen solutions.

Following the procedure used in the studies on antigens, absorption experiments were employed to confirm the identification of the immobilization antibody. The standard G antiserum was diffused against a G antigen extract that had previously been incubated with antiserum at 12° C. for 24 hours. Nine sera which immobilized G animals in two hours when used in dilutions of 1 : 200 or greater successfully absorbed the antigen responsible for the band found in G antigen-antibody systems. Three with poor G titers and 26 without any G titer when used as absorbents had no effect on the appearance or position of the band.

A final corroborative group of experiments was carried out using the mutual dilution method. A single serum having a high immobilization titer against G animals and precipitating antibody capable of withstanding several-fold dilution was chosen as a standard serum. When eight anti-G sera were mutually diluted with the standard serum, it was found that the antibody restricted to the G antigen-antibody reaction and present in all systems involving these sera was either related to or identical with the antibody found in the standard G antiserum, the band formed being reinforced upon the addition of the sera being tested. Additional mutual dilutions among the eight sera confirmed this finding.

Although the evidence for the identity of the band characteristic of homologous systems with the immobilization antigen and antibody is convincing, there appears to be a rather poor correlation between the titer of a serum as determined by immobilization tests and the concentration of precipitating antibody. Thus, serum P#16 with about the same G immobilization titer as sera P#23, P#2, and P#12 has less than 25% of the precipitating antibody of these antisera (Table I). Other "exceptional" sera are P#4 and P#21 which possess precipitating antibody and yet do not immobilize. It is apparent, then, that immobilization antibody and precipitating antibody, although closely correlated, are not identical. The serological nature of the relationship of the two kinds of antibodies is being studied. As would be expected, preliminary absorption experiments have demonstrated that it is possible to remove all precipitating antibody from certain antisera without abolishing all immobilizing activity.

As for the immobilization antigens, although these studies have demonstrated that in animals of serotype G one particular precipitating antigen is found and in animals of serotype C it is not found, but a second precipitating antigen is, it is not known whether immobilization antigens and precipitating antigens are identical. It is possible that only a portion of the immobilization antigen is capable of precipitation or that there may be precipitating antigen that does not take part in immobilization.

Summary

1. A study of serotypes G and C, of variety 2, *Paramecium aurelia*, has been made, using diffusion in agar. It has been shown that animals of each serotype have a specific precipitating antigen which is lacking in animals of other serotypes. Consequently, it seems probable that these antigens are the immobilizing antigens.

2. Comparisons of antibody concentration, as determined by immobilization titers and by band position, show that the precipitated band may not be composed

solely of immobilizing antigen and antibody, and that there is precipitating antibody that is not capable of immobilizing.

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OOGENESIS IN HABROTROCHA TRIDENS (MILNE)

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The bdelloid rotifers of about 200 species, classified into 19 genera and 4 families, reproduce exclusively by parthenogenesis, males being unknown in this group. It is therefore interesting to study the behavior of their chromosomes during oogenesis. I have reported such a study on one of them, *Philodina roseola* (1956). Some of the findings reported in that paper are as follows: 1. In *Philodina roseola,* there are two maturation divisions, both equational. 2. No indication of synapsis has been observed between any two of the chromosomes. Individual chromosomes even in the earliest oocytes were observed to be in a condensed state. The anaphase chromosomes of the oogonial division do not despiralize in forming the nuclei found in the syncytial ovary. The chromosomes, after the last oogonial division, remain condensed, and, by progressive packing together, they form first a ring and then a homogeneous and spherical mass of chromatin occupying the center of the nucleus. When one of these nuclei is isolated by the ovary to form an oocyte, its condensed chromosomes do not despiralize into leptotene threads, but persist in a condensed state. As the germinal vesicle increases in size they separate from each other until finally 13 condensed chromosomes can be easily counted. 3. The zygoid chromosome number in this rotifer is 13. Three of the 13 chromosomes, two dot-shaped ones and one that is appreciably longer than the rest, are morphologically distinguishable from one another and from any one of the other ten (Fig. 37). It was suggested that the chromosomes in this group of animals may have lost their homology.

The present paper reports observations made on *Habrotrocha tridens,* which belongs to the family of Habrotrochidae. For methods employed in this study reference may be made to my paper dealing with *Philodina roseola.*

Observations

As in *Philodina roseola,* the ovary and its accessory structure, the vitellarium, in *Habrotrocha tridens* are syncytial. In mature animals, the ovary consists of about 30 nuclei, with the chromosomes in each nucleus grouped so tightly together that they form a single spherical body of smooth outline. When one of the nuclei is isolated by the ovary to form an oocyte, the individual chromosomes do not go through the meiotic changes characteristic of oocytes in other animals. They simply remove themselves from each other as contracted bodies while the nucleus increases in size.

Figure 1 illustrates the condition of the chromosomes in the nucleus of a young oocyte. At this stage, it is still difficult to differentiate the individual chromosomes. But as the nucleus enlarges, the chromosomes begin to stand out clearly as condensed bodies. If the whole history of the chromosomes in the developing egg is

not studied carefully, the thread-like structures of a less basophilic character in the nuclei illustrated in Figures 2 and 3, for instance, may be erroneously interpreted as leptotene threads, and the intensely stained true chromosomes in a condensed state regarded as heterochromatic sections of thread-like chromosomes. But as the oocyte and the germinal vesicle increase in size, the true chromosomes become more and more separated from each other and more easily differentiated tinctorially from the thread-like material. The true situation can be clearly seen in Figures 2–9. The stage of maximum growth of a germinal vesicle is seen in Figure 10. At this stage the nuclear sap appears in fixed material as a fine-meshed net. Thirteen chromosomes are spread out widely apart from each other and can be most easily counted at this stage. Two of them are appreciably smaller than the others and are dot-shaped. These two, however, are not of equal size. It will be recalled that in *Philodina roseola* there are also two dot-shaped chromosomes of unequal size. But unlike *Philodina roseola*, this form does not possess among the remaining 11 chromosomes one that is conspicuously longer than the rest.

Further development from this stage is indicated by a shrinking in mass on the part of the germinal vesicle; the nucleus thus becomes reduced in size and irregular in shape (Fig. 11). But as this takes place, the nuclear sap seems to react differently to the fixative. The fine-meshed appearance no longer prevails, and threads begin to make their appearance within the nucleus (Figs. 12–16). At this stage there is a difference between the present form and *Philodina roseola*. In the latter, the nucleus keeps on decreasing in size to a much more extreme degree, and finally becomes again rounded in outline; whereas in *Habrotrocha tridens*, the nucleus stops shrinking much earlier, and I have not observed any well-rounded germinal vesicle of reduced size (Figs. 15–17). On the contrary, when the nucleus has decreased in size to the extent shown in Figure 17, it begins to break up. Figure 17a gives the condition of the same nucleus at a lower level of focus than the one at which Figure 17 was drawn. At this level of focus, the nuclear membrane shows unmistakable signs of disintegration. After the membrane is broken, the chromosomes are set free in the cytoplasm (Figs. 18 and 19). In *Philodina roseola* the chromosomes next spread out into a more or less irregular line pressed close to the wall of the oocyte. This line formation has not been observed in the present form, and Figure 19 represents the distribution of the chromosomes most frequently observed at this time of development.

At first the cytoplasm immediately surrounding the free chromosomes does not appear any different from that seen in any other area within the egg. But when the chromosomes have pulled away from the periphery of the oocyte and have become more separated from each other, they are seen to be embedded in an area of cytoplasm which appears to be more vacuolated than the rest of the cytoplasm in the developing egg (Fig. 20). This was also observed in *Philodina roseola*. Then, also as in *Philodina roseola*, it seems that under the influence of the chromosomes, a homogeneous and light-staining material is developed in which the chromosomes are embedded (Fig. 21). It is within the area occupied by this material that the spindle is developed later. There seems to be a change going on in this material, as a result of which the area formerly occupied by the homogeneous material now appears to be traversed by threads. These threads are not as taut and trim in outline as the regular spindle fibers (Fig. 22). Such stages have been frequently observed, and in some cases the fibers do give a rather close resemblance to the regular

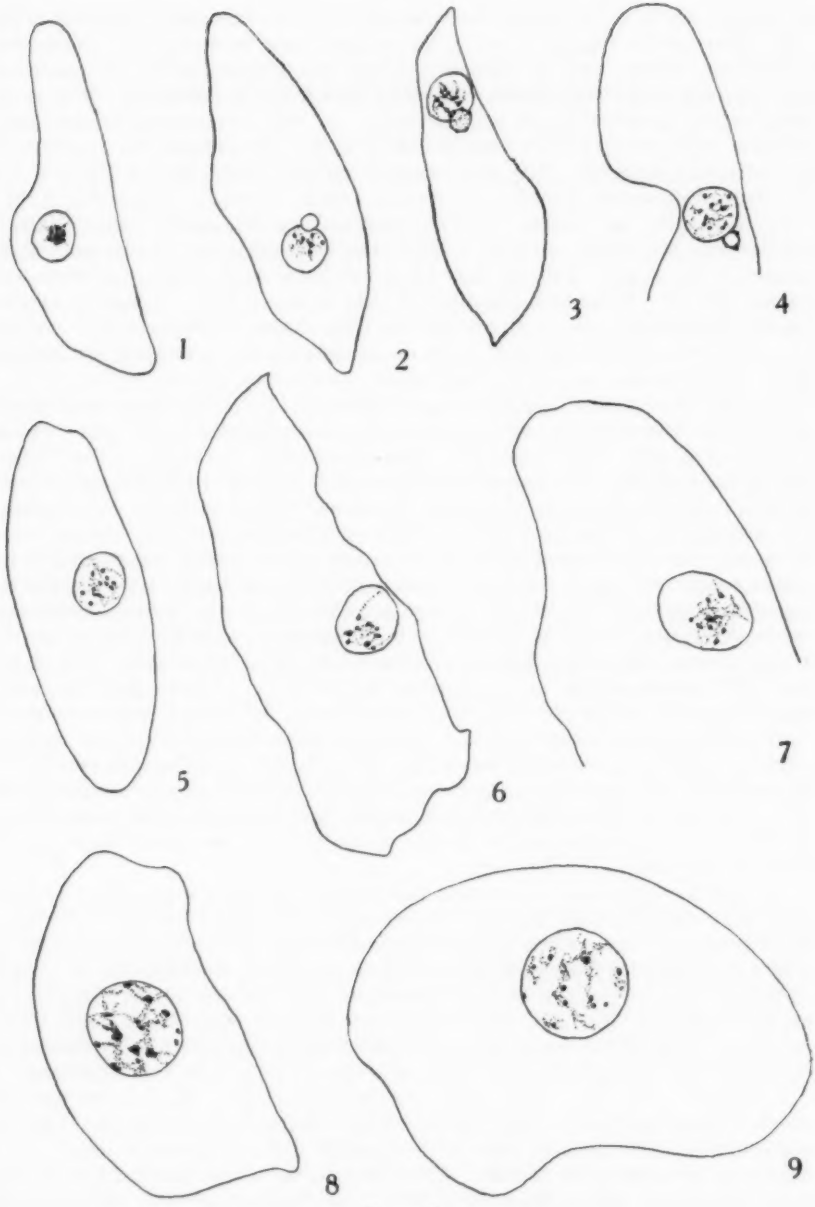

PLATE I

spindle fibers. In *Philodina roseola*, the next stage has been found to be a monopolar spindle which eventually develops into an orthrodox bipolar one (Hsu, 1956). But in *Habrotrocha tridens*, I have seen a single tripolar spindle which may be described as a compound structure formed by three different bipolar spindles, the long axes of which all lie on the same plane and so arranged with regard to each other that the structure forms a somewhat triangular configuration. The chromosomes form three separate equatorial plates, one on each component spindle (Fig. 23). Unfortunately, this is the only one I have observed in my slides.

Another spindle, also the only one I have found in my material, is represented in Figure 24. There are two cones placed at an angle as shown. The chromosomes are gathered loosely at the general area toward which the truncate ends of the two cones converge. The chromosomes are not all in one level of focus. But due to the rarity of these spindles, it is simply unsafe to consider them definitely as structures normal in *Habrotrocha tridens*. They should be merely recorded and left for future discussion when more evidence becomes available. However, in view of the peculiar spindle and its manner of development observed in *Philodina roseola* where evidence was more abundant, the possibility that the two peculiar spindles observed in *Habrotrocha tridens* may express normal stages of development in this rotifer cannot be entirely excluded. If these spindles be considered as normal structures, I should then think that they represent stages following those depicted in Figures 21 and 22 and preceding that represented by Figure 25. I would assume that Figure 24 shows a stage in which the compound spindle is breaking up and a bipolar structure is in the process of forming. This process would consist of a disintegration of the base spindle in Figure 23 and a movement on the part of the chromosomes. Then a proper rotation of the two remaining cones shown in Figure 24 would produce an orthodox bipolar spindle such as that shown in Figure 25. Of course, this is largely a conjecture.

Whatever may be the true significance of these two peculiar spindles, there is no question that a bipolar spindle does finally form to effect the first maturation division in *Habrotrocha tridens*. Figures 25, 26, 27 and 28 represent lateral and polar views of the first maturation division. In Figure 29, we see two anaphase groups of chromosomes which demonstrate beautifully that this division involves no reduction in chromosomes. Figure 30 shows a polar-body and the chromosomes within the secondary oocyte being regrouped to form the metaphase plate of the second division. Figures 31 and 32 both show the metaphase spindle of the second division, each with a polar-body directly over it at a higher level of focus (Figs. 31a and 32a), which fact indicates that the long axes of the spindles of the two divisions are perpendicular to each other. The number of chromosomes which could be made out in each one of these two metaphase spindles, counting each dumb-bell shaped granule as a unit, indicates that the second division is also equational in *Habrotrocha tridens*. Usually in such cases something like 10 to 11 units could be

All figures are camera lucida drawings made at 1500 ×

PLATE I

FIGURES 1–9. Oocytes showing the condensed chromosomes in their germinal vesicles, and becoming progressively more easily distinguishable from the less intensely stained thread-like structures as the oocytes mature. In Figures 2, 3 and 4, an idiozome is shown in contact with the germinal vesicle.

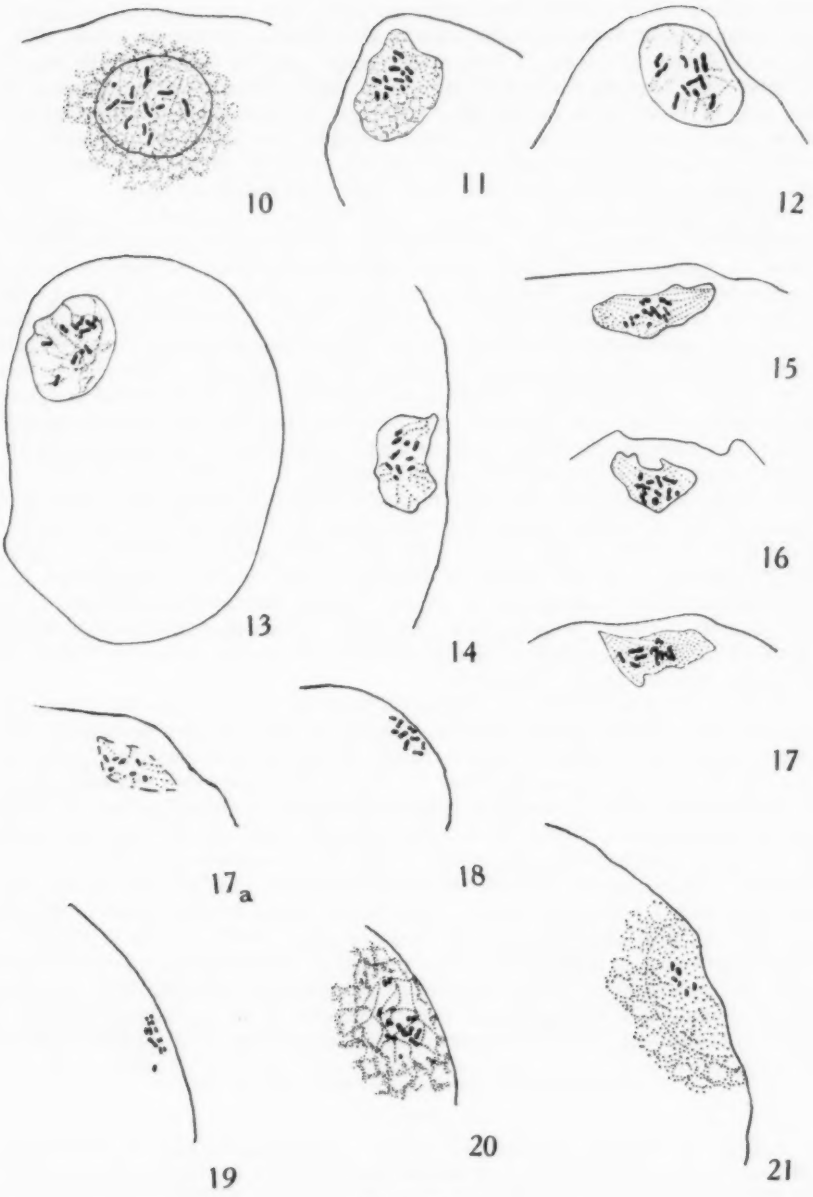

PLATE II

counted. Having seen many eggs of about this stage, I cannot help feeling that the second division takes place immediately after the first without giving the chromosomes enough time to be included within a nucleus. In *Philodina roseola,* however, a metabolic nucleus is achieved between the two divisions.

After the two maturation divisions, the comparatively large nucleus of the mature egg goes into a resting stage in which the chromosomes lose their staining intensity (Fig. 33). Figure 34 is a polar view of the metaphase plate of the first cleavage division in which 13 chromosomes with two relatively smaller ones are clearly visible. As in *Philodina roseola,* during anaphase of the first few cleavage divisions of the embryo, "elimination bodies" are visible (Fig. 35).

Discussion

In both *Philodina roseola* and *Habrotrocha tridens,* the oocyte undergoes two maturation divisions, and the zygotic chromosome number, 13, is maintained in the mature egg because both these divisions are equational. No sign of chromosome pairing has been observed. I have examined as yet too few species to venture an opinion on the question as to whether or not all the species of Bdelloidea follow this pattern of oogenesis. I can only point out the fact that the two species examined belong to two different families of Bdelloidea.

In view of the genetic principles which should apply to ameiotic parthenogenetic animals, we should not be surprised to find in their chromosomes evidence of aneuploidy, polyploidy, structural rearrangement and the loss of diploid character in both the genetic and the cytological sense. In this connection I cannot do better than to quote White (1954) (p. 341): "In ameiotic parthenogenesis genetic segregation will not occur. Recessive mutations and structural rearrangements will tend to accumulate indefinitely in such organisms, only the ones which are immediately deleterious being eliminated by natural selection. Such forms must consequently be expected to become gradually more and more heterozygous, but all the offspring of a single female will resemble their mother exactly, except for newly arisen dominant mutations and differences due to the action of the environment. An ameiotic form evolving for a long period of time might be expected eventually to lose its diploid character in both the genetic and the cytological sense, its two chromosome sets having become almost completely unlike. Moreover, since no

Plate II

Figure 10. A germinal vesicle of full growth in which the thread-like structures have disappeared and a fine-meshed net has made its appearance. Note the 13 chromosomes: two dot-shaped, the rest all dumb-bell shaped indicating doubleness.

Figure 11. A germinal vesicle beginning to shrink, exhibiting an irregular outline.

Figures 12–17. Germinal vesicles of increasingly reduced size in which the fine-meshed net is in turn replaced by fibers.

Figure 17a. The same germinal vesicle as depicted in Figure 17 but at a lower level of focus, showing signs of disintegration of its membrane.

Figures 18 and 19. Chromosomes lying free in the cytoplasm close to the wall of the oocyte.

Figure 20. Chromosomes have pulled away from the cell wall and become more scattered in an area of material which clearly appears to be more vacuolated than the cytoplasm elsewhere in the egg.

Figure 21. Chromosomes lying in an area of light-staining material in which short sections of fiber can be vaguely seen.

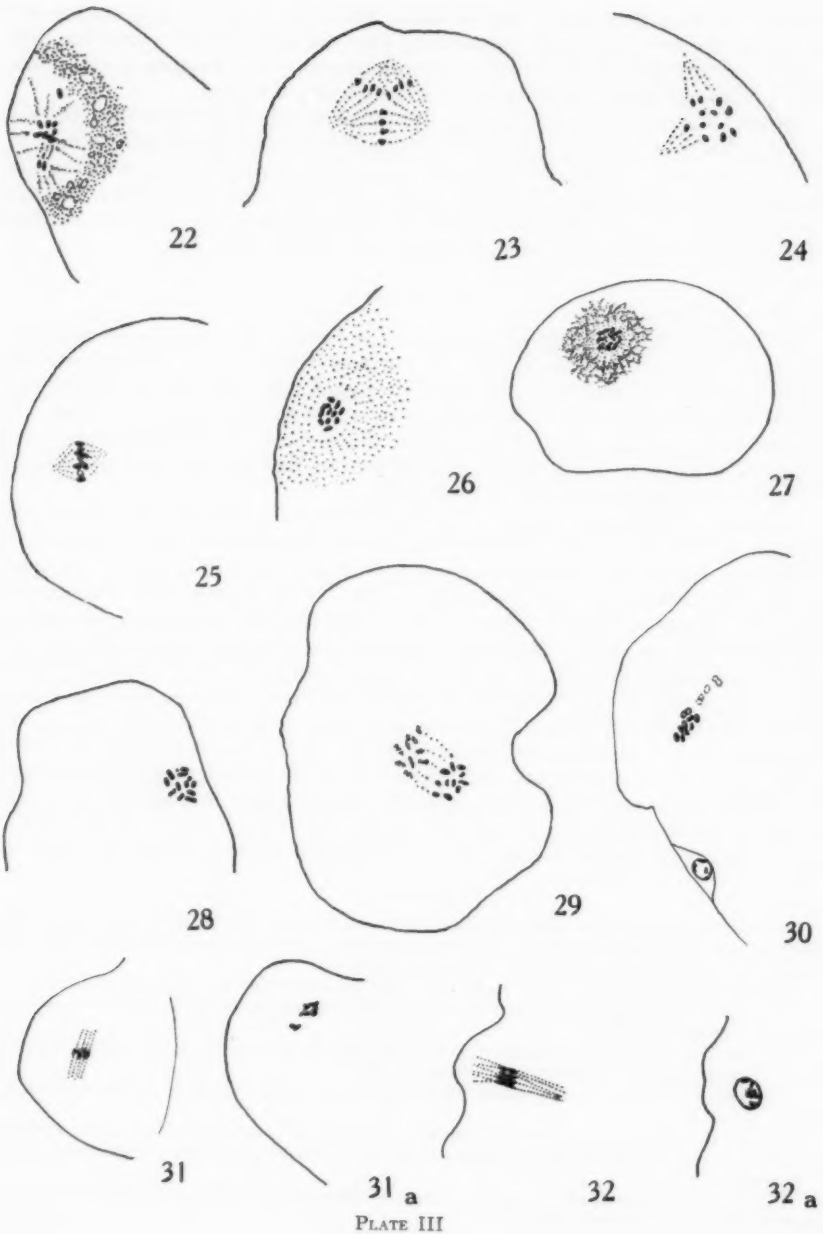

PLATE III

pairing of chromosomes takes place during the maturation of the eggs, there is no 'mechanical' barrier to the establishment of any type of polyploidy in such forms and various forms of aneuploidy, due to irregular reduplication of some chromosome elements, must be expected to occur."

It seems to me that at least three chromosomes in *Philodina roseola* may very well have been involved in structural rearrangement of some kind, though not necessarily just among themselves (Fig. 37). This conclusion should hold unless we assume that the bisexual ancestor of this form had one pair of dimorphic chromosomes and another one without a mate. But this seems to me unlikely. Besides, although reports on chromosome number in rotifers are very confusing, none of them besides the two forms under discussion has been reported to possess an odd number of chromosomes (Makino, 1951) (p. 11). It would seem, then, that the odd number of chromosomes seen in the two species of Bdelloidea under discussion may indicate aneuploidy due either to irregular reduplication of some chromosome elements or some such structural rearrangements as centric fusion accompanied by the loss of one chromosome.

It is difficult to say whether or not the two dot-shaped chromosomes were originally members of the same homologous pair. But since they are present in both *Philodina roseola* and *Habrotrocha tridens,* and since there is no other chromosome that is comparable to them in morphology, it may be safe to look upon them as originally forming a pair. In that event, their disparity in size and the fact that the smaller one of the two, especially in *Philodina roseola,* often stains less intensely than the bigger one could perhaps be regarded as indications of loss of homology between them.

Turning next to the chromosome which in *Philodina roseola* is conspicuously longer than the rest, I must say I cannot confidently identify it in *Habrotrocha tridens.* This is the only morphological difference I can point out with confidence between the chromosome complexes of these two forms.

It should perhaps be stressed here that the absence of pairing of chromosomes in these two forms should not be interpreted necessarily as evidence of loss of homology on the part of their chromosomes, since according to the genetic principle applying to ameiotic parthenogenetic organisms the loss of the diploid character between homologous member chromosomes is possible precisely because of asynapsis.

PLATE III

FIGURE 22. Chromosomes in an area in which the light-staining material is replaced by coarse fibers reaching between the chromosomes and connecting them to the boundary of this area.

FIGURE 23. A compound spindle consisting of three bipolar spindles, each with its own equatorial chromosome plate.

FIGURE 24. A compound spindle disintegrating.

FIGURE 25. Lateral view of a first polar spindle.

FIGURES 26–28. Polar view of three equatorial plates of the first polar division.

FIGURE 29. A mitotic figure at anaphase of the first maturation division.

FIGURE 30. The first polar-body and the chromosomes within the secondary oocyte regrouping to undergo the second maturation division.

FIGURE 31. A metaphase spindle of the second maturation division.

FIGURE 31a. Chromosomes belonging to the first polar-body seen at a higher level of focus directly above the metaphase spindle represented in Figure 31.

FIGURES 32–32a. The same as Figures 31 and 31a except in this case the first polar-body nucleus is already formed.

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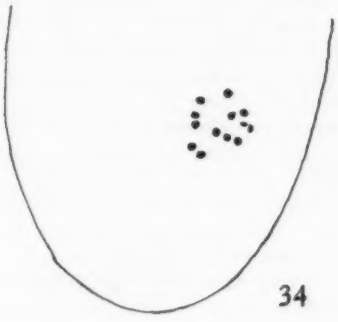

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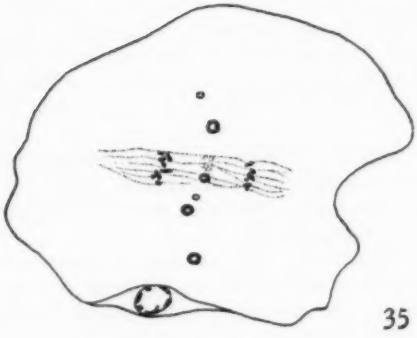

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PLATE IV

In other words, asynapsis is here supposed to be antecedent to the loss of homology. Moreover, the persistent condensed state of the chromosomes in my material complicates the situation, since our current theory explaining pairing of chromosomes takes into account, besides the singleness of the threads, also the degree of their uncoiling. In this connection we should, of course, recall that in Neurospora, MacClintock (1945) has reported pairing of relatively contracted chromosomes. Incidentally, it may be mentioned that since daughter chromosomes in these forms can separate without difficulty, the coiling which their chromonemata assume must be of the paranemic type, using the term which Sparrow, Huskins and Wilson (1941) have proposed.

With regard to the first polar spindle, it must be said that due to the paucity of observations the situation in this rotifer is not clear. It is difficult to venture an opinion as to whether or not a tripolar spindle represents a normal stage of development. More observations are needed before a reliable answer can be given. At present, I can only say with confidence that the two spindles shown in Figures 23 and 24 are very distinct and unmistakable structures. Although I have not made an attempt to study the mitochondria condition in *Habrotrocha tridens,* I feel quite certain that mitochondria are not involved in this case, Devisé (1922) and Junger (1931, 1934) notwithstanding. The three separate equatorial plates of chromosomes, one on each spindle, ought to settle the question.

This research was supported by the National Science Foundation and the Washington State Initiative 171 Research Fund for Biology and Medicine. I am indebted to my colleague, Dr. W. Thomas Edmondson, for identification of the rotifer used in this investigation, and to Miss Lorraine Pilon for efficient technical assistance.

Summary

1. The pattern of chromosome behavior during egg formation in *Habrotrocha tridens* is the same as that found in *Philodina roseola.* The oocytes undergo two maturation divisions, both equational.

2. The zygoid chromosome number is 13, the same as that of *Philodina roseola.*

3. The pair of dot-shaped chromosomes of unequal size is found in each of these forms, though the conspicuously longer one seen in *Philodina roseola* (Fig. 37) cannot be identified in the present form (Fig. 36).

Plate IV

Figure 33. A portion of a mature egg with a comparatively large nucleus about ready to undergo the first cleavage division. Note the two polar-bodies.

Figure 34. Polar view of the equatorial plate of the first cleavage division. Note the 13 chromosomes, two of which are appreciably smaller than the rest.

Figure 35. An anaphase figure of the first cleavage division. Note the polar-body and the "elimination bodies."

Figure 36. One late prophase and four metaphase chromosome plates seen in the embryonic cells of *Habrotrocha tridens.* Note the two dot-shaped chromosomes.

Figure 37. Two metaphase chromosome plates seen in the embryonic cells of *Philodina roseola.* Note the two dot-shaped chromosomes and the one that is conspicuously longer than the rest.

4. No sign of synapsis has been observed in either form.

5. The chromosomes exist in a condensed condition in the nuclei of the ovary after the last oogonial division, and remain condensed throughout at least the first maturation division.

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SELECTIVE LIGHT ABSORPTION BY THE LENSES OF LOWER VERTEBRATES, AND ITS INFLUENCE ON SPECTRAL SENSITIVITY

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Visual processes in all vertebrates apparently depend upon a group of closely similar carotenoid-proteins. Since the spectral distribution of sensitivity is determined by the absorption spectra of these pigments, it is no accident that most vertebrates are sensitive to approximately the same band of wave-lengths. In man, this range lies between the rough limits of 400 mμ and 700 mμ. As an expression of these limitations, we have come to call wave-lengths longer than 700 mμ "infra-red" and those shorter than 400 mμ "ultra-violet."

The long-wave-length limit of sensitivity is relatively inflexible among vertebrates, because the visual pigments so far isolated from retinas do not absorb significantly above 700 mμ. At the other end of the spectrum, however, the limit imposed is of quite a different sort. The visual pigments rhodopsin, porphyropsin and iodopsin (Wald, 1955; Wald, 1939; Wald, Brown and Smith, 1955) all show considerable absorption between 300 and 400 mμ, with a secondary maximum present in this region. In the case of rhodopsin, the absorption at 360 mμ is nearly 30% of that at the 500 mμ maximum; the absorption at 600 mμ, by contrast, is less than 10%. Light of these "ultra-violet" wave-lengths is thus potentially available for utilization in the sensory process.

In the case of human vision, Wald (1952) has shown that the short-wave-length limit at approximately 400 mμ is imposed by selective absorption in ocular tissues. Below 310 mμ, in the region of general protein absorption, almost all light is absorbed by the cornea, since it is the first tissue encountered. The lens, which appears yellow in color (especially in aged persons), is an effective cut-off filter for radiations below 400 mμ. Aphakics (persons with lenses excised for cataracts) tested by Wald could read an optometrist's chart by the isolated 365 mμ line from a mercury arc, under which conditions a normal person could see nothing at all. Although the pigment responsible for the coloration of the human lens—and for its properties as a filter—has not been definitely identified, the indications are that it is a melanin (Gourevitch, 1949).

Walls and Judd (1933) and Walls (1940) attempted a comparative survey of the occurrence of such filtering lenses in other vertebrates. They found yellow lenses in the eyes of some diurnal reptiles (snakes and certain geckoes), lampreys, squirrels, tree shrews and ground squirrels (*Citellus*). None were seen in fishes

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or amphibians; but the yellow perch and the bowfin (*Amia*) both had yellow corneas. Walls believes that the functional advantage in the selective removal of short-wave-length radiations lies in promoting visual acuity. Chromatic aberration, since it increases exponentially as the wave-length decreases, can produce serious errors in the violet and ultra-violet (Wald and Griffin, 1947). It is therefore adaptive for diurnal animals—whose requirement is for acuity and not sensitivity—to remove this region of the spectrum before the light reaches the retina. This idea is supported by Walls' finding that yellow lenses occur only in diurnal forms.

The mere presence of a yellow coloration, however, does not mean that the lens is a successful ultra-violet filter. Many yellow pigments (for example, xanthophyll and carotene) absorb in the blue region of the spectrum and not in the near ultra-violet. Conversely, lack of a visible yellow color does not mean that the lens is not an effective filter for the near ultra-violet. A filter absorbing nothing above 400 mμ but cutting off sharply at 390 mμ, for example, would appear colorless to the human eye, but it would be a powerful aid to visual acuity for the animal possessing it.

The fact that the human eye is a poor instrument with which to assess these properties prompted the present investigations. These experiments are an attempt to measure quantitatively the selective absorption of lenses, comparing those of a wide variety of lower vertebrates. Such measurements may clarify the functional significance of this interesting visual adaptation; in addition, they provide concrete information about an important dimension of sensory capacity in these animals. Some studies on the chemical basis of this selective absorption are also reported. Finally, the influence of these filtering lenses upon spectral sensitivity is assessed by electrophysiological measurements comparing ultra-violet sensitivity in animals with and without their lenses. A preliminary report of some of these experiments has appeared elsewhere (Milkman and Kennedy, 1955).

Methods

For measurements of intact lens absorption, fresh lenses were dissected from the experimental animals and placed in a holder designed to fit a spectrophotometer cuvette. The holder was constructed in such a way that light passed through the lens and out an exit pupil of approximately one mm. diameter, corresponding in position to the central axis of the lens, and thence to the photocell of the spectrophotometer. Thus the absorption of only a small axial section of lens tissue was measured. The lens and holder were immersed in a cuvette filled with mineral oil, which was chosen to match as closely as possible the refractive index of the lens and thereby eliminate errors due to refraction. The mineral oil also prevented the lens from growing opaque during the period of measurement.

A Beckman DU quartz spectrophotometer was used to measure the transmission of the lenses to light of different wave-lengths. Measurements were checked repeatedly, and it was found that the transmission at a particular wave-length did not change significantly during the course of an experiment. A "blank" was used which consisted of an adapter without the lens, suspended in a similar medium. In certain species, the transmission of the cornea was measured using the same apparatus and a similar technique.

In attempts to discover the chemical basis of the selective absorption found in the lenses of certain of the species tested, aqueous extracts were made from large amounts of homogenized lens tissue. Lenses were ground with distilled water in a glass mortar; protein material was removed, either by dialysis, precipitation by heating, or making up the solutions to 50% ethanol.

Extracts were chromatographed on Whatman No. 1 filter paper in butanol-acetic acid-water mixtures (5:1:4), using ascending strips or cylinders of paper. They were viewed under ultra-violet light from a mercury arc lamp, equipped with a filter which removed almost all visible wave-lengths. The chromatograms were treated in various ways. Some were sprayed with ninhydrin; in others, the spots were cut out and eluted with a small volume of water. All absorption spectra were measured in a Beckman spectrophotometer. In some cases, it was desirable to obtain absorption spectra directly from spots on the paper; these were measured directly in the spectrophotometer, using a "blank" of dry butanol-saturated paper and employing a photo-multiplier attachment for extra sensitivity. Satisfactory spectra could be obtained in this way under conditions when eluted samples might have been too dilute to yield satisfactory measurements.

In order to measure directly the effect of selective lens absorption on spectral sensitivity, the spectral sensitivity function of intact frogs was compared with that of animals deprived of their lenses. Briefly, the technique involved recording the electroretinogram (the slow action potential of the retina) from either the cornea or, in the case of excised eyes, from the vitreous body. Moist cotton wick electrodes were used with a capacity-coupled pre-amplifier and oscilloscope. Monochromatic light produced through interference filters (or, in the case of 365 mμ, by isolation of that mercury arc line) was directed onto the eye of the preparation through a pair of opposed annular wedges which regulated the intensity. The optical system was calibrated with a thermopile and sensitive galvanometer. In each experiment, a certain amplitude of b-wave (the large positive potential of the electroretinogram) was chosen as the "criterion response." The intensity of constant-duration flash necessary to produce a response of this amplitude was then found for each wave-length, and the reciprocals of these "threshold" intensities were plotted as a spectral sensitivity curve. Frogs were curarized and dark-adapted before each experiment. Each wave-length was then tested in turn, with control flashes frequently interspersed to assure that a constant level of sensitivity was maintained. Then the lens was removed and the experiment repeated. In a number of experiments, excised eyes were used instead of intact animals; this procedure proved equally satisfactory.

Results

The lenses of many of the fish and amphibians studied showed marked filtering properties. Representative absorption curves of intact lenses are shown in Figure 2, with Wald's data on the Rhesus monkey lens included for comparison. The species studied are divided into roughly three groups. Members of the first of these possess lenses which, like the human lens, show a cut-off at about 400 mμ, but they are clearly better filters in that their rise in extinction is sharper. As a result of their lack of absorption above 400 mμ, these lenses do not appear yellow. The group includes the yellow perch (*Perca flavescens*), the calico bass (*Pomoxis sparoides*), and the blue-gill sunfish (*Lepomis pallidus*), all common fresh-water

species; the scup (*Stenotomus versicolor*), the summer flounder (*Paralichthys dentatus*), the rudderfish (*Serolia zonata*), and the sea robin (*Prionotus evolans*), all marine species; and the grass frog (*Rana pipiens*). The absorption spectra of lenses in this group are roughly similar (Fig. 1), except for that of the frog, in which the cut-off occurs at a definitely shorter wave-length. This will be discussed below.

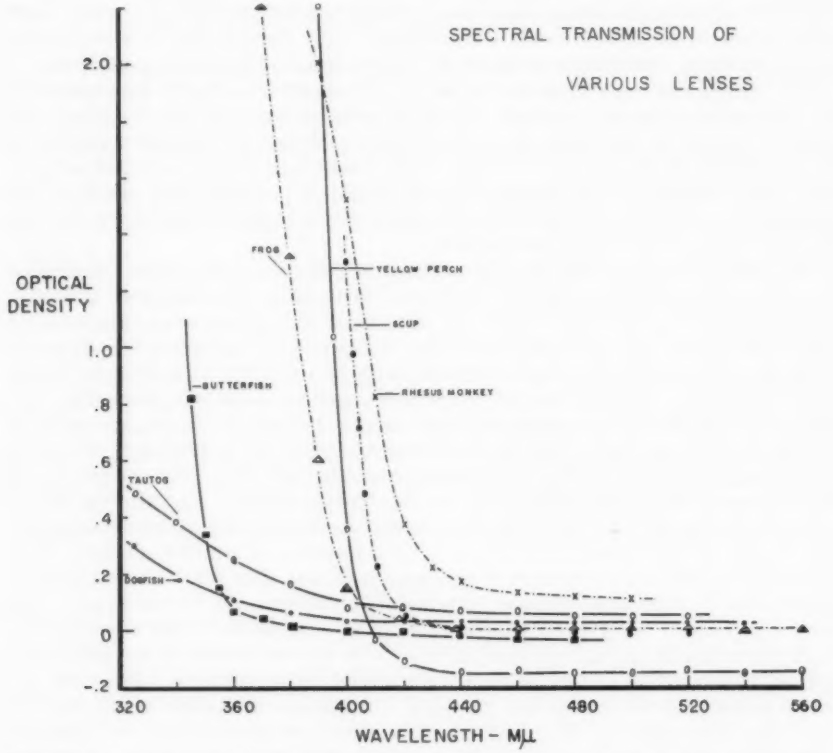

FIGURE 1. Measurements of the spectral transmission of lenses from various lower vertebrates. Data on the lens of the Rhesus monkey from Wald (1949) are included for comparison.

The butterfish (*Poronotus triacanthus*) shows instead a steep rise in lens extinction near 350 mμ, and thus is in a group by itself.

A third group, represented by the tautog (*Tautoga onitis*), the smooth dogfish (*Mustelus canis*) and the toadfish (*Opsanus tau*), all marine bottom species, and the catfish (*Amieurus nebulosus*), a fresh-water bottom scavenger, appear to have only a gradual, slight rise in lens extinction down to 320 mμ. The brook trout (*Salvelinus fontinalis*) is also in this category, but possesses a cornea which shows strong absorption beginning at 400 mμ.

Aqueous extracts of all lenses tested belonging to the first group showed absorption spectra similar to that given in Figure 2, a preparation from lenses of the cod (*Gadus callarias*). Flounder, rudderfish perch, calico bass and sea robin preparations were also tested and found to be spectrally identical; in most further studies, cod lenses were used because they could be obtained in large quantities, fresh, through the courtesy of the Booth Fisheries Corporation of Boston.

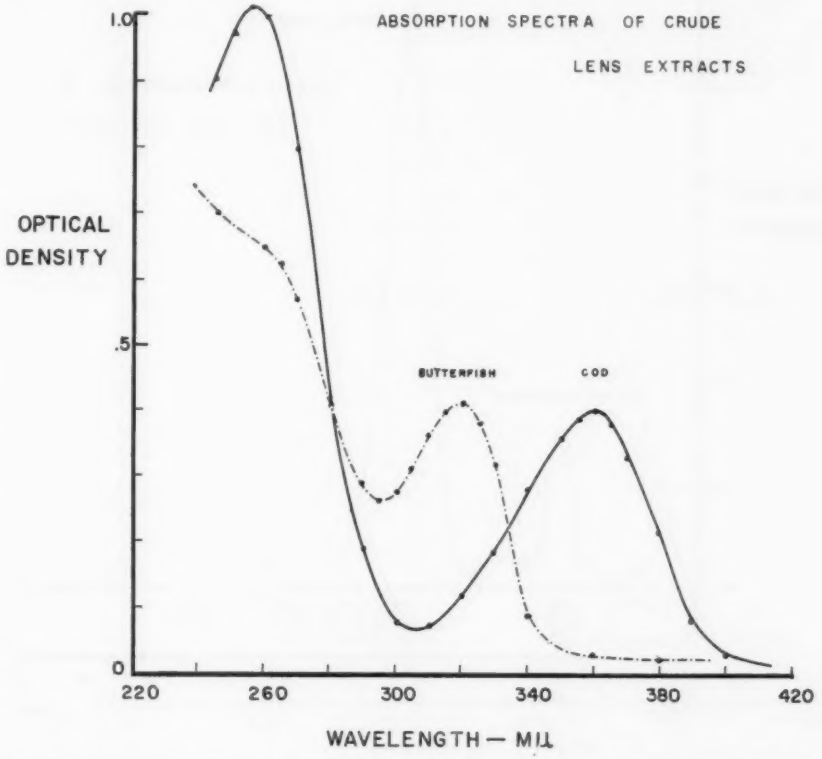

FIGURE 2. Absorption spectra of aqueous extracts from lenses of the butterfish (dotted line) and the cod (solid line). Maxima have been adjusted to same height.

The absorption spectra of these extracts, as shown in Figure 2, show a maximum at 360 mμ, and this absorption band is responsible for the action of the lens as a cut-off filter. In the case of the frog, similar extracts had their maxima at 345 mμ, consistent with the slight displacement of the extinction of intact frog lenses.

Extracts of butterfish lenses (Fig. 2) had absorption maxima at 320 mμ, which explains the fact that intact lenses in this species have their steep rise in extinction at 350 mμ instead of near 400 mμ. No absorption bands between 300 mμ and

400 mμ were found in extracts from lenses which lacked the steep rise in extinction (third group).

The term "pigment" is usually restricted to those substances which absorb in the *human* visible range. This range, however, is restricted by the presence of an ultra-violet-absorbing lens; it is appropriate in this context to refer to the visual

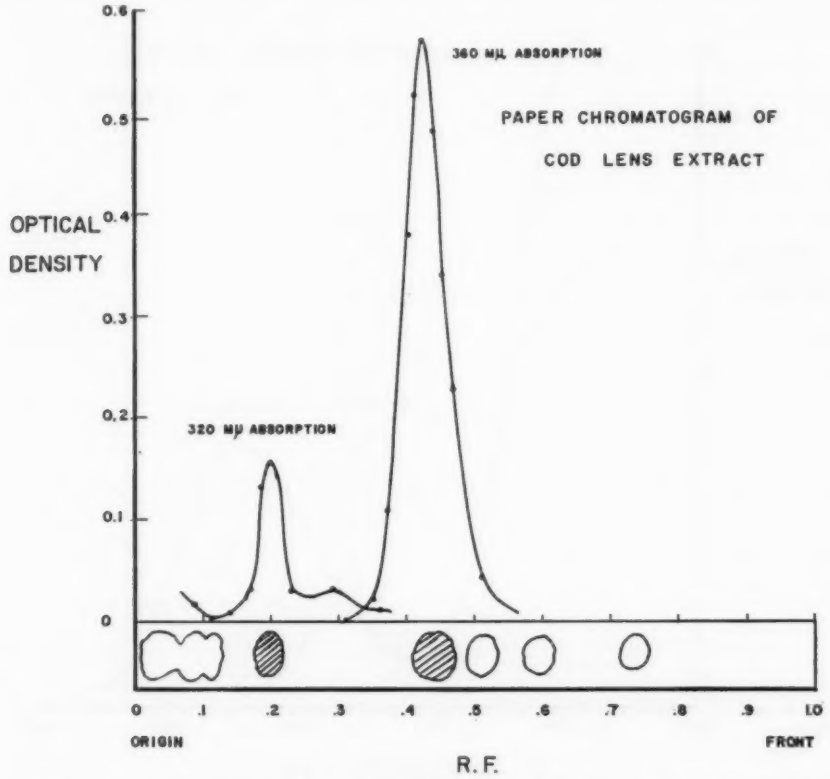

FIGURE 3. Chromatography of cod lens extract. The shaded spots at R. f. 0.19 and 0.43 are, respectively, the presumed oxidation product of 360-pigment and 360-pigment itself: the first spot corresponds with a peak of absorption measured at 320 mμ, and the second with a peak of absorption measured at 360 mμ.

range of vertebrates in general as extending from 310 mμ, below which all ocular tissues absorb strongly, to 700 mμ, the upper limit of visual pigment absorption, provided no special intra-ocular filters intervene. In this sense, then, the filtering substances of the fish lens qualify as pigments, since they absorb light which is visible—though not to humans. We therefore will refer to these substances as pigments, labeling them specifically by their absorption maxima: for example, 360-

pigment for the substance extracted from lenses in the first group, and 320-pigment for the material isolated from butterfish lenses.

After it was found that selective absorption by these lenses had a specific chemical basis, some attempts were made to characterize the substances responsible. Both 360-pigment and 320-pigment are water-soluble, somewhat soluble in methanol and ethanol, and insoluble in all organic solvents tried. They are stable in acid (pH 1), but in alkali (pH 12) they break down slowly and their characteristic absorption bands disappear.

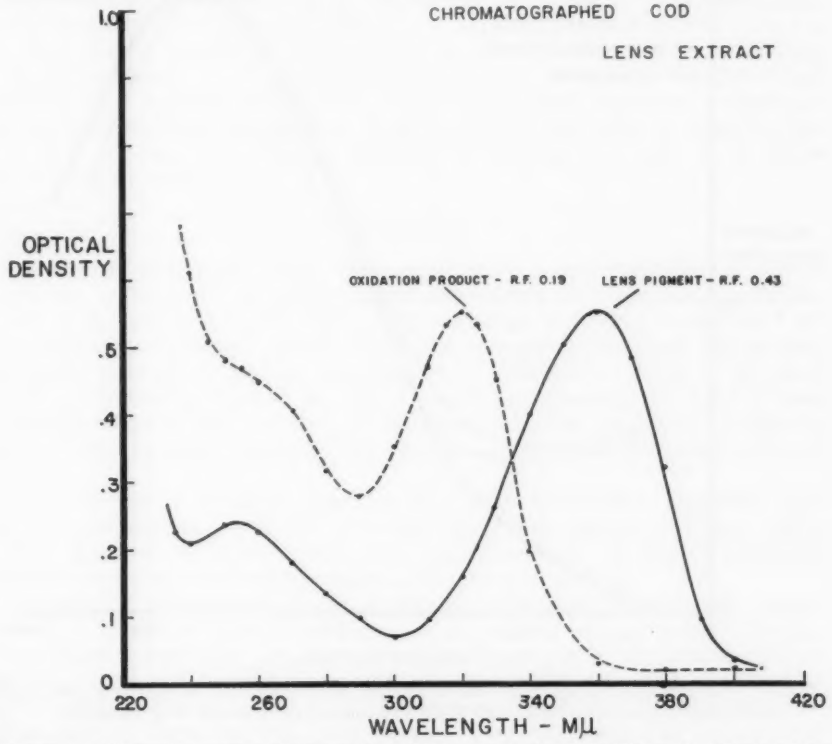

FIGURE 4. Absorption spectra of eluates from the two spots shown in Figure 3. Maxima have been adjusted to the same height.

360-pigment is apparently readily oxidized on standing, or by bubbling oxygen into the solution. The absorption maximum at first shifts from 360 mμ to 320 mμ; this latter band later disappears, and the final product shows only a rising general absorption into the ultra-violet, often developing a tan color suggesting the formation of a melanin-like polymer.

Paper chromatography of lens extracts in butanol-acetic acid-water mixtures (5:1:4) reveals a series of fluorescent and ninhydrin-positive spots. Presumably, a variety of amino acids and polypeptides is present, together with other sub-

stances such as riboflavin. Figure 3 shows the presence of 360-pigment as a ninhydrin-positive, non-fluorescent spot at R. f. 0.43; another spot, yellow-fluorescent and ninhydrin-positive, is present at R. f. 0.19.

Concentrated lens extracts were then chromatographed in streaks, and the bands at R. f. 0.43 were cut out and eluted with distilled water to yield a quantity of purified 360-pigment. When such eluates were allowed to stand overnight at room temperature and rechromatographed, two spots were seen: one was identical

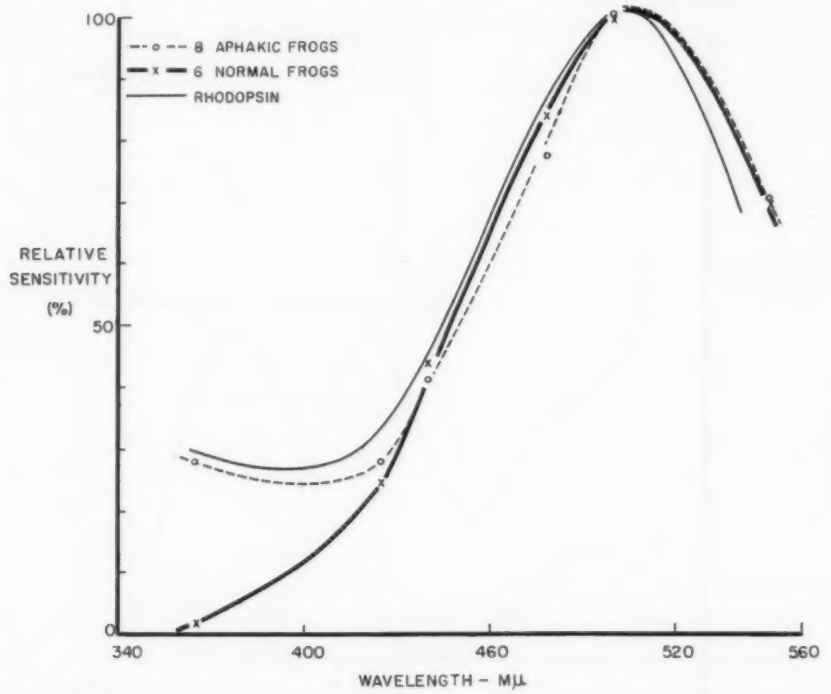

FIGURE 5. Average spectral sensitivity curves at short wave-lengths for normal frogs and frogs with lenses excised, compared with Wald's absorption spectrum for frog rhodopsin.

in position and absorption spectrum to the original eluted spot, and a second was present at R. f. 0.19. This spot was ninhydrin-positive and yellow-fluorescent, and had an absorption maximum at 320 mμ. This is the presumed oxidation product of 360-pigment; as can be seen from Figure 4, it is spectrally similar to 320-pigment from the butterfish.

The ninhydrin-positive nature of both substances in these experiments indicates that an amino group is found in 360-pigment and its derivative. The absorption spectra of chromatographically purified 360-pigment and of its derivative indicate that a second absorption band at 225 mμ is characteristic of both, though in the latter it is present as a shoulder.

When iodine is added to 360-pigment in solution, a quantitative shift of the absorption maximum to 320 mμ is produced. The conversion apparently produces a single product, since there is a clear isosbestic point. It is not certain whether the observed shift is due to saturation of a conjugated double-bond system or another sort of oxidation. The product is spectrally identical with 320-pigment from the butterfish and with the previously-described derivative of 360-pigment; 320-pigment from the butterfish will not react with iodine.

The pigment from the frog lens has an absorption maximum at 345 mμ. It differs from the 360-pigment not only spectrally, but in that it will not add iodine and is acid-unstable and alkali-stable.

The effect of selective lens absorption upon the spectral sensitivity of the frog is shown in Figure 5. The spectral sensitivity function of animals with their lenses removed is in satisfactory agreement with the absorption spectrum of rhodopsin down to 365 mμ in the ultra-violet. Intact frogs, however, begin to show low sensitivity at 425 mμ, and at 365 mμ, sensitivity is only approximately 1% of that at the 500 mμ maximum.

Discussion

It appears from these results that a great many lower vertebrates, as well as mammals, possess an intra-ocular system for filtering out ultra-violet radiations which might otherwise impair visual acuity. A rough correlation is observed here, too, between the existence of such filters and an apparent ecological requirement for acute vision on the part of their possessors. The species found to lack such filters are bottom-feeders like the dogfish which rely primarily on other sensory systems in their feeding. Active, surface-living forms all seem to have filtering lenses; in the butterfish, however, the lens transmits a considerable band of ultra-violet.

The correlation is rather better among higher vertebrates. Squirrels, tree-shrews and primates, among the mammals, are largely diurnal, and have yellow lenses; no nocturnal animal has been found to possess one, and Weale (1953) has shown that the cat lens has a high transmission down to 400 mμ.

In gauging the adaptive value of an intra-ocular filtering mechanism in aquatic animals, a number of complications must be considered. First, though it is generally believed that ultra-violet light penetrates water poorly, the transmission of water for near ultra-violet (350 mμ–400 mμ) is actually quite high compared to light of 550 mμ–600 mμ (Jerlov, 1951). Second, the presence of suspended material increases scattering to a great degree. The consequences of this fact are difficult to ascertain: scattering increases exponentially with decreasing wavelength, so that the presence of suspended matter selectively increases the extinction of short-wave-lengths. However, there are some secondary considerations which cannot be ignored: a plankton-feeding fish, for example, might use ultra-violet sensitivity advantageously to locate concentrated areas of suspended matter (including organisms) by short-wave-length light scattered from them.

Walls (1942) has advanced arguments to support the idea that filtering lenses are a sort of evolutionary "second line" in the battle against chromatic aberration. Retinal cone oil droplets are held to be the usual method of filtering short-wavelength radiations. These are found in turtles and birds (yellow, red, orange and

colorless), amphibians (yellow) and some fishes (colorless). Walls believes that a group which becomes nocturnal in the course of evolution loses its oil droplets; the filtering lens is evolved as a substitute in secondarily diurnal forms. This, he believes, explains the presence of yellow lenses in snakes and diurnal geckoes.

This idea does not seem to explain the situation adequately. Wald and Zussman (1938) have shown that the oil droplets of birds contain three carotenoid pigments, a different one of which is responsible for each color. The yellow one is xanthophyll, which is also responsible for the yellow coloration of the human *macula lutea*. Such droplets are unquestionably filters, but they are not filters designed for removing the ultra-violet. Xanthophyll, for example, has its absorption maximum near 450 mμ, and has declined to a very low absorption in the region of 400 mμ where chromatic aberration begins to become especially serious. The other oil-droplet pigments have their maxima at even longer wave-lengths. In the human eye, in which the cone-rich fovea is equipped with a xanthophyll filter, there is also a yellow lens.

Other evidence, too, contradicts the idea that the filtering lens is functionally identical with the oil droplets and replaces them when the latter are lost in evolution. The frog, which has been shown in these experiments to possess a sharp ultra-violet cut-off in its lens, *also* has yellow cone oil droplets. Finally, we have made observations on the yellow cornea of the yellow perch, and find that it owes its coloration to the presence of (primarily) β-carotene, a carotenoid with nearly the same absorption spectrum as xanthophyll. The perch, too, has a lens filter for ultra-violet. It thus appears that the carotenoid filters in the eyes of vertebrates (oil droplets, yellow corneas, and *macula lutea*) either serve some special function unrelated to the selective absorption of ultra-violet by the lens, or that they are accessory filters which serve to widen the band of short-wave-length absorption. In either case, they are not adaptively equivalent to an ultra-violet filter in the lens.

Evidence suggests that the pigment of the primate lens is a melanin (Gourevitch, 1949). Yellow lenses of other mammals may also owe their coloration to a melanin, although little chemical characterization has been done. The pigment can be extracted by alkali, but not by water (Walls, 1940).

The lenses of the fishes and amphibians studied here owe their selective absorption to an entirely different sort of pigment, which is water-soluble. It has not been possible so far to determine the chemical identity of these lens pigments; their behavior suggests, at least, that 360-pigment from a variety of fish and 320-pigment from the butterfish are closely related chemically.

Not many groups of water-soluble natural compounds show the type of ultra-violet spectrum exhibited by these substances. The two major groups which do are the pteridines (Forrest and Mitchell, 1954a, 1954b, 1955) and some metabolites of tryptophane such as kynurenine. There are chemical similarities between the lens pigments and these two classes of substances, but also some marked differences. At present, there is no definite basis for deciding in which group of compounds the lens pigments belong, although pteridines have been previously isolated from the eyes and integument of fish (Pirie and Simpson, 1946; Hüttel and Sprengling, 1943).

The data on frog spectral sensitivity at short wave-lengths show clearly the large effect which selective lens absorption has on actual visual processes. How-

ever, there is a quantitative discrepancy between *in vitro* measurements of lens absorption and the electrophysiological sensitivity data. Spectrophotometric measurements on the excised frog lens show that it has a transmission at 365 mμ of less than 1% of the incident light. Comparison of the relative sensitivity of normal and aphakic frogs at 365 mμ, however, reveals that the normal animals are about 5% to 10% as sensitive as those lacking lenses. The differences may be explained if it is remembered that in the spectrophotometric measurements, only a small central core of lens tissue was measured; thus, this figure represents the extinction of the longest optical path through the lens. In the intact, dark-adapted animal, with its pupil dilated, light passes through the edges of the lens as well, thus reducing its effectiveness as a filter and accounting for the difference in sensitivity.

The experiments show that the scotopic spectral sensitivity function of frogs clearly agrees with rhodopsin absorption down to 365 mμ, provided no intra-ocular filters intervene, and that an estimate of the effect of such filters in modifying spectral sensitivity may be made by measuring their transmission *in vitro*.

Summary

1. Spectrophotometric studies of fresh intact lenses from a variety of fish and from frogs have shown that they are steep cut-off fibers for ultra-violet radiations, selectively absorbing almost all light of wave-length shorter than 400 mμ.

2. Certain species of fish possess lenses having high transmission in the near ultra-violet, between 320 and 400 mμ; these species must be sensitive to this spectral region, since visual pigments absorb there. Lenses of the butterfish show a steep cut-off at about 350 mμ.

3. There appears to be a correlation between possession of ultra-violet filtering lenses and a requirement for acute vision, supporting the idea that they aid visual acuity by eliminating wave-lengths which produce severe chromatic aberration. Such lenses, however, cannot be regarded as functionally equivalent to such intra-ocular carotenoid filters as retinal oil droplets and *macula lutea* since they absorb in quite different spectral regions. The theory that lens filters are an evolutionary "replacement" for oil droplets in secondarily diurnal animals is thus not in agreement with these findings.

4. Substances responsible for the properties of these lenses as filters have been extracted with water from the lens tissue. Lenses which cut off at 400 mμ yield a substance with an ultra-violet absorption maximum at 360 mμ; those of the butterfish, which cut off at 350 mμ, yield a substance with maximum absorption at 320 mμ. A presumed oxidation product of 360-pigment has been obtained which is spectrally similar to 320-pigment from the butterfish. Both substances have been characterized as to solubility, ultra-violet absorption spectra, and chromatographic behavior, but no definite identification has been made.

5. Comparison of spectral sensitivity in normal frogs and frogs deprived of their lenses has been made by recording the electroretinogram. The results show that the lens has the anticipated effect in restricting short-wave-length sensitivity. In frogs without lenses, scotopic sensitivity is in good agreement with the absorption of rhodopsin down to 360 mμ, while in normal animals sensitivity declines sharply below 400 mμ.

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RELATIVE INTENSITY OF OYSTER SETTING IN DIFFERENT YEARS IN THE SAME AREAS OF LONG ISLAND SOUND

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Quantitative studies of marine bottom invertebrates have been conducted since the early part of the century, and the results have substantially enriched our knowledge and understanding of aquatic communities. The contributions of many workers to this important branch of marine biology have been reviewed by several authors, including Spärck (1935) and, more recently, Sanders (1956).

Regardless of the progress made, there still remains one aspect of this field which has been relatively neglected but which should be of special interest to many students of bottom communities. In general, it concerns the recruitment of the new year-classes of such forms as mollusks and echinoderms that have pelagic larvae which, after a free-swimming period, descend to the bottom and metamorphose, the act commonly called setting. In particular, it deals with the variations in the intensity of setting of the same species in the same area in different years, and comparing these variations with those of other, nearby areas.

Our long-term studies of the biological events of Long Island Sound give us the opportunity to discuss certain aspects of this problem in relation to the American oyster, *Crassostrea virginica*. The conclusions are based on data collected during the past 12 years, 1944 through 1955, from ten chosen areas. The locations of these areas, which we shall call stations, are shown in Figure 1. They were confined to three depths— 10, 20 and 30 feet—and represented three major oyster-producing sections of Long Island Sound, namely, New Haven, Milford and Bridgeport. The combined area of these sections is approximately 80 square miles.

The intensity of setting at each of the stations was evaluated by counting the number of recently set oysters on special collectors consisting of wire mesh bags filled with old oyster shells (Prytherch, 1930). This is the standard method in use at our laboratory for over 20 years, and with which most oyster biologists and oystermen are now well familiar (Loosanoff and Engle, 1940; Loosanoff, Engle and Nomejko, 1955). It is important to emphasize that the locations of the stations remained the same during the 12 years, and that the methods of determining the intensity of setting were identical for all stations.

To evaluate the relative productivity of each station, we employed a simple ranking method by giving, each year, Rank 1 to the most productive station, Rank 2 to the next most productive, and so on, until the least productive was given Rank 10. For example, for 1944, Station 1, the most productive, was given Rank 1; Station 2, the least productive, Rank 10; Station 3, Rank 9; etc. (Table I).

To determine the relative productivity of the stations during the entire 12-year period, we expressed the rank of each station as the sum of its yearly ranks (Table I). Naturally, the stations that were generally better producers and, therefore,

entitled to low ranks, such as Station 9, showed lower sums than the stations that were less productive. On the basis of this total score, we gave a long-range rank to each station.

The question immediately arose as to whether these ranks would remain approximately the same if the stations were graded for their performance only during the years of better sets, namely, 1944, 1945, 1946, 1953 and 1955. However, our analysis showed that the ranks of the stations for these years were not substantially different from the long-range ranks based on the 12-year observation period (Table I).

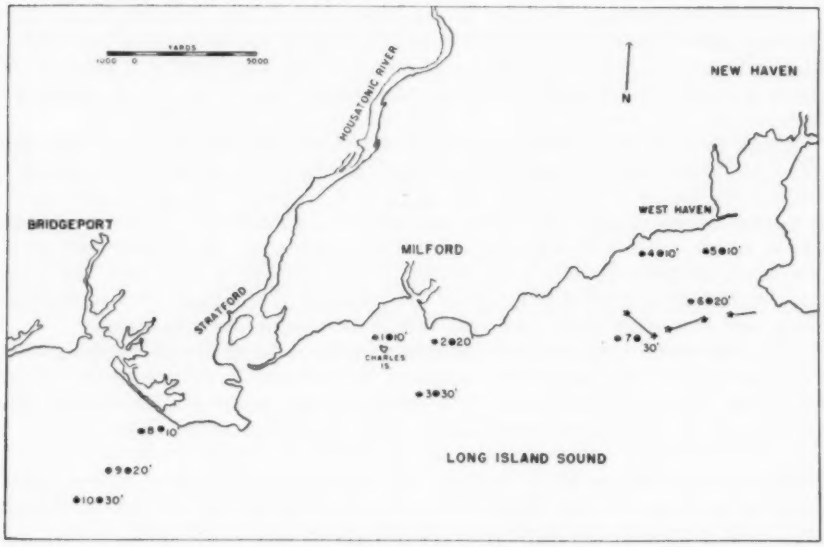

FIGURE 1. Locations and depths (in feet) of ten stations established for observation of oyster setting in Long Island Sound, 1944–1955.

A close study of the data provided information as to the relative importance of the stations, the depths, and the areas in the different years. It was established that, excluding Stations 2, 3 and 4, each station ranked first at least one year out of 12. It was also found that a 10-foot station ranked first, five times; a 20-foot station ranked first, three times; and a 30-foot station, four times. Thus, considering that at the 10-foot depth we have one station more than at 20 or 30, it appears that Rank 1 was occupied by stations of each of the three depths the same number of times.

If we add the sums of the yearly ranks of all the stations at the same depth and then calculate the average sum of the yearly ranks of these depth-groups, we will find that the 10-foot stations have a score of 65.2; 20-foot stations, 57.3; and 30-foot stations, 75.7. Thus, the 20-foot stations seem to be generally somewhat more productive than the others, while the 30-foot stations appear to be the least pro-

ductive of the groups at all three depths. The latter conclusion, however, may not be well founded because the low rank of the 30-foot stations is chiefly due to the history of setting at Station 3 which, through the 12 years, was consistently one of the poorest, never rising above sixth place; whereas another 30-foot station, Number 10, was, in more than half the instances, among the five best stations, and ranked first on three occasions (Table I).

TABLE I

Rank order of the sampling stations representing oyster-setting areas of Long Island Sound during the 12-year period, 1944–1955

Areas	Milford			New Haven				Bridgeport		
Stations	1	2	3	4	5	6	7	8	9	10
Depth in feet	10	20	30	10	10	20	30	10	20	30
Years										
1944	1	10	9	5	2	4	7	6	8	3
1945	1	4	8	10	6	5	9	2	3	7
1946	6	9	8	10	5	1	3	7	2	4
1947	7	6	8	9	5	2	10	1	3	4
1948	8	6.5*	10	3	6.5*	5	2	4	1	9
1949	8	4	9	3	5	7	1	2	6	10
1950	7	9	10	8	4	6	2	5	1	3
1951	9	10	7	8	4	6	5	3	2	1
1952	1	5	10	7	4	6	8	3	2	9
1953	7	3	8	10	4.5*	6	9	4.5*	2	1
1954	7	2.5*	9.5**	9.5**	1	5	6	8	4	2.5*
1955	4	5	6	10	7	9	8	3	2	1
Sum of 12 yearly ranks	66	74	102.5	92.5	54	62	70	48.5	36	54.5
Long-range rank, 12 years	6	8	10	9	3	5	7	2	1	4
Rank for 1944, 1945, 1946, 1953, 1955	3	7	9	10	5	6	8	4	2	1

*, ** Indicate a tie between stations for the same rank.

The data were also used to evaluate the relative productivity of the three areas under observation, namely, New Haven, Milford and Bridgeport (Fig. 1). By adding the ranks of all the stations, as given in Table I, for each area and year, and dividing the resulting figure by the number of stations, the average station rank for each area was determined (Table II). Accordingly, each area was given a yearly rank, the one with the lowest score occupying the first or most productive position. The sums of the average yearly ranks for each year, as shown at the bottom of Table II, were also determined. It was found that the Bridgeport area occupied first rank, or the best producing position, nine years out of 12, and never held third or last place. The New Haven area was next, occupying first rank for

three years. The Milford area, however, never reached the highest position, ranked second only four times, and was in the third, or lowest position for the remaining eight years.

We cannot offer a fully satisfactory explanation for the variations or, in some instances, stability from year to year in the relative productivity of our stations. Such considerations as original number of eggs discharged; mortality of larvae due to diseases, enemies, or lack of food; and several others are, of course, of impor-

TABLE II

Average yearly station-ranks of the New Haven, Milford and Bridgeport areas, and general rank of each of these three areas for each year of 1944–1955 period. Sums of average yearly ranks of stations of each area, and the ranking of the areas during the entire 12-year period are also given

Years	Areas			Ranks		
	New Haven	Milford	Bridge-port	1	2	3
1944	4.50	6.67	5.67	New Haven	Bridgeport	Milford
1945	7.50	4.33	4.00	Bridgeport	Milford	New Haven
1946	4.75	7.67	4.33	Bridgeport	New Haven	Milford
1947	6.50	7.00	2.67	Bridgeport	New Haven	Milford
1948	4.13	8.17	4.67	New Haven	Bridgeport	Milford
1949	4.00	7.00	6.00	New Haven	Bridgeport	Milford
1950	5.00	8.67	3.00	Bridgeport	New Haven	Milford
1951	5.75	8.67	2.00	Bridgeport	New Haven	Milford
1952	6.25	5.33	4.67	Bridgeport	Milford	New Haven
1953	7.38	6.00	2.50	Bridgeport	Milford	New Haven
1954	5.38	6.33	4.83	Bridgeport	New Haven	Milford
1955	8.50	5.00	2.00	Bridgeport	Milford	New Haven
Sum of average yearly ranks	69.64	80.84	46.34	Bpt.—9 yrs. N. H.—3 yrs. Mfd.—0 yrs.	N. H.—5 yrs. Mfd.—4 yrs. Bpt.—3 yrs.	Mfd.—8 yrs. N. H.—4 yrs. Bpt.—0 yrs.

tance. Nevertheless, there is little doubt that the intensity of setting of oysters at all stations depends to a large extent upon the peculiarities of the inshore system of water currents.

The complexity and characteristics of these currents in the oyster-producing section of Long Island Sound are still relatively unknown because no detailed study has ever been made. We know, however, that planktotrophic larvae, with comparatively longer pelagic lives, like those of oysters and many other pelecypods, are carried by water masses and that their distribution is controlled by the currents. Under certain conditions the direction of the currents may be so changed that the larvae will be carried away from the areas where setting normally takes place, and eventually perish. In other instances, as reported by Coe (1953) for *Donax gouldi*, an enormous increase in the population of a species may occur because swarms of pelagic larvae, about ready to set, are unexpectedly brought in-

shore by water currents. Hence, it is understandable that the productivity of small areas, such as those designated for our stations, should, in general, be more affected by minor changes in larvae-carrying currents than that of larger areas, such as New Haven, Milford or Bridgeport, which cover many square miles of oyster-producing bottom, and should certainly display more stability in maintaining their relative positions.

These observations emphasize the importance of studying the different aspects of local minor currents, including their direction, velocity and stratifications. They also indicate the importance of understanding the relationship between the behavior of such currents and the locations of the spawning beds, of oysters or other mollusks, where the larvae originate.

Our studies suggest, moreover, that minor currents are often extremely precise in their behavior. This was well demonstrated by observations on the intensity of setting of oysters at our Station 10, located several miles from the shore and in comparatively deep water, but where, nevertheless, heavy setting continued steadily day after day for as long as three or four weeks because the currents consistently brought a supply of ready-to-set larvae to that point. In 1955, this regularity was not noticeably affected even by the strong winds of hurricane "Connie" nor by the winds and record-breaking floods of "Diane." Finally, they imply the potential danger of interfering with established combinations of the favorable ecological conditions existing on the bottom by modifying its contour so as to change the directions of the local currents. Although these changes may not affect commonly studied factors, such as temperature and salinity, they may, nevertheless, so alter the currents that the larvae will be carried to new areas, some of which may not be suitable for their setting.

We wish to express our appreciation to Barbara J. Myers for her assistance in analyzing these data, and to our colleagues, Harry C. Davis and Rita S. Riccio, for their helpful suggestions in preparing this paper.

Summary

1. During the 12 years of observations none of the stations, representing relatively small bottom areas, always occupied a position among the best oyster set producers.

2. If larger areas instead of individual stations were compared, a definite tendency of the Bridgeport area to be more productive than the others was evident.

3. There was no evidence that the stations located at a definite depth, such as 10, 20 or 30 feet, consistently produced better sets of oysters than the stations at other depths.

4. There may be a great variability in the density of oyster set even within a given depth and district in the same year. For example, Stations 4 and 5, although located in the same district and at the same depth, showed a rather different standing with Long-Range Ranks of 9 and 3, respectively.

5. Local minor water currents are important in the relative productivity of bottom areas.

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INFLUENCING THE CALLING OF SEA ROBINS (PRIONOTUS SPP.) WITH SOUND[1, 2, 3]

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Despite abundant evidence that fishes hear and produce sounds (Fish, 1948, 1954; Griffin, 1955; von Frisch, 1938), a review of the literature (Moulton and Backus, 1955) on attempts to influence fish movements with man-made sounds has uncovered reports only of quickened movements of fishes during production of such sounds. Nor has a biological significance of any sound known to stem from a fish, whether produced by stridulation of skeletal parts or by the air bladder, been clearly demonstrated. However, the apparent relationship between sound-production by some species of fishes and their respective breeding seasons has been noted by a number of authors (Fish, 1954, pp. 51, 83; Goode, 1888, p. 137; Marshall, 1954, p. 254), and the possible significance of fish calls in bringing individuals of the same species together has been suggested. Sounds are also produced during defensive spine raising of such fishes as groupers, grunts, squirrel fishes and sea robins.

During the summer of 1954, it was accidentally discovered that the production of certain fish calls, later identified as the calls of sea robins, could be stimulated by transmission of certain sounds into the water, and that the calling could be suppressed by other sounds (Moulton, 1955). The study resulting from this finding was pursued further during the summer of 1955. The observations yielded evidence that calls produced during the breeding season of two species of sea robins (*Prionotus carolinus* L. and *P. evolans* L.) are produced as responses to calls of the same species, and that by the transmission of appropriate sounds, some degree of control over the calling of sea robins may be exercised.

The sound-generating equipment employed in the experiments here described consisted of a Hewlett-Packard audio oscillator Model LAJ or a Magnecorder tape recorder Model PT6J; either an Altec Type A-323B or a Craftsman Model C550 amplifier, and a QBG transducer. The monitoring system was an AX-120 ADP or an AX-58-C Rochelle salt hydrophone and a Woods Hole Suitcase amplifier. Recordings were made on the Magnecorder tape recorder at a speed of 15 in./sec. and were analyzed on a Vibralyzer vibration analyzer. The experiments were performed from a raft anchored over 72 feet of water in Great Harbor, Woods Hole, Massachusetts.

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[2] The work was performed while the author was a Research Fellow at the Woods Hole Oceanographic Institution during the summers of 1954 and 1955.

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The Sounds of Sea Robins

The sound-producing air bladder of the sea robin has been described by Fish (1954). It is the apparent source of two different calls. One of these calls is a vibrant grunt produced when a sea robin is handled in or out of water, and when a sea robin is brought to the surface by net or by hook and line. The grunt accompanies fin erection.

As determined by vibration analysis, the sea robin grunt is a single burst of noise lasting about $\frac{1}{10}$ second. The upper frequency limit is approximately 1.7 kc., the lower below 44 cps. The grunt is audible to the unaided ear above the water when a sea robin is submerged four feet beneath the surface. Noises of frequency characteristics similar to those of the grunt may be obtained by pressing the air bladder through the ventral body wall of the intact fish, and by stimulation of the nerves to the drumming muscles located on the lateral surfaces of the bilobed air bladder.

The onset of the breeding season of the sea robins at Woods Hole is marked by the production by these fishes of a staccato call. Although this call has been monitored from sea robins contained within a live car at the surface of Great Harbor, no single fish has been identified as the source of an individual call. This call is not produced under conditions that bring forth the grunt already described, and it is much more frequently produced by fishes in the Harbor than by caged specimens.

The breeding season of the sea robins at Woods Hole extends from June to September, with July and August the height of the season (Bigelow and Schroeder, 1953). In 1955, listening began on 29 June. The first staccato calls were heard on 5 July, and calls were heard on each day of listening thereafter until work terminated for the summer on 30 August. The number of outbursts of calling and the number of calls comprising a single outburst increased rapidly during the first half of July (compare Tables I and II). During the latter part of August calling became more infrequent.

Table I

Responses of sea robins to audio oscillator signals in Great Harbor, 15 July, 1955

Time	Number of signal transmissions	Response
1415	1	2 calls
1420	1	No calls
1430	1	No calls
1440	1	No calls
1450	1	No calls
1500	2	1 call after first signal
1510	3	1 call after first signal
1520	2	No calls
1530	2	No calls
1540	2	1 call after first signal
1550	3	1 call after second signal
1600	2	No calls
1610	4	1 call after signals one and two
1620	3	No calls
1630	3	No calls

TABLE II

Responses of sea robins to recordings of the staccato call and to audio oscillator signals, Great Harbor, 20 July, 1955

Time	Number and type of signal transmission	Response
1425	1 recording	2 calls
1430	1 signal	No calls
1435	1 recording	No calls
1440	1 signal	No calls
1445	1 recording	4 calls
1450	1 signal	No calls
1455	1 recording	No calls
1500	1 signal	2 calls
1505	1 recording	No calls
1510	1 signal	No calls
1515	1 recording	No calls
1520	1 signal	6 calls
1525	1 recording	No calls
1530	1 signal	No calls
1535	1 recording	No calls
1540	1 signal	No calls
1545	1 recording	No calls
	(5 spontaneous calls during this interval)	
1550	1 signal	No calls
1555	1 recording	No calls
1600	1 signal	No calls
1605	1 recording	No calls
	(12 spontaneous calls during this interval)	
1610	1 signal	No calls
1615	1 recording	No calls
1620	1 signal	No calls
1625	1 recording	No calls
1630	4 signals	1 call after No. 2
1635	3 recordings	No calls
1640	3 signals	No calls

The staccato calls consist of pulses of noise usually produced in pairs, at an average rate of 22 pulses/second. The paired arrangement of the pulses in a typical call is probably due to a slightly asynchronous contraction of the drumming muscles on the two lobes of the air bladder. The pairing of the pulses is not distinguishable to the ear, but can be seen on vibration analysis. Absence of the double pulses in a portion of some calls and, rarely, throughout a call suggests that one lobe of the air bladder may be silent during sound production by the other lobe.

The individual pulses of the staccato call lie between 500 cps and 4 kc. The frequencies of greatest intensity lie between 700 cps and 2.5 kc. The respective intensity peaks of the paired pulses are at different frequencies on the vibragrams, separated by approximately 1 kc. This is presumably related to a differential resonance of the two air bladder lobes which generally differ somewhat in size. It is possible to obtain sounds of similar frequency and intensity characteristics by palpitation of the dissected air bladder.

Influencing Production of the Staccato Call

With the sound-generating equipment employed, it is possible to transmit a series of sound pulses crudely imitative of the staccato call of the sea robin when the audio oscillator is set at 17 to 40 cps. (The QBG emits a considerably distorted wave train when driven with a sine wave at these frequencies.) With transmissions timed to correspond to the duration of an average call, 2½ to 3 seconds, production of the staccato call by free sea robins was repeatedly incited during July and August of 1954 and 1955.

Tables I and II present the results of two experiments extending over 2 hours and 15 minutes on 15 and 20 July, 1955. On 15 July (Table I) from one to three imitations of the staccato call were transmitted at ten-minute intervals, except for the second trial which followed the first by five minutes. Of the 15 trials, 6 were followed immediately by calling of free sea robins. There was no calling during the listening period other than immediately following signal transmissions.

On 20 July (Table II), playing of recordings of the sea robin staccato calling into the harbor water was alternated with transmission of imitations of the calling at five-minute intervals. During the 28 tests of 20 July, the calling of free sea robins was heard five times immediately following transmissions, twice after playing recordings of the calling and three times following transmissions of the imitations. Two spontaneous outbursts, frequent by 20 July, were heard during the period of the experiment. As Table II also indicates, outbursts of several calls were the rule by 20 July, whereas earlier in the month single or double calls comprised the characteristic outburst in 1955.

Suppressing of the Staccato Call

Signals of 200 to 600 cps transmitted for the approximate duration of a staccato call interrupt the production of this call by sea robins. (Again the QBG signal is considerably distorted as at the lower frequency.) Signals above 2 kc. have never been effective in suppressing the calling. Signals from 600 cps to 2 kc. are variable in effectiveness. Sea robins confined in a live car and sea robins on the bottom of Great Harbor, observed by an aqualung diver, Mr. Robert Weeks of the Woods Hole Oceanographic Institution, show no obvious change in behavior during transmission of signals effective in suppressing the staccato call.

Discussion

Conditions bringing forth the grunting of sea robins suggest that this sound is part of a general alarm reaction. It may be of value in nature as an adjunct to the spiny armor of the species in discouraging enemies, but no evidence is available on this point.

That the staccato calling reaches its climax near the peak of the sea robin breeding season is strongly suggestive of a relation of this calling to breeding activities, and the possibility cannot be overlooked that the calls serve as a species recognition device in waters where visibility is rather poor. Mr. Robert Weeks has informed me that visibility on the bottom of Great Harbor beneath the raft was a little over 6 feet on 10 July, 1955, and that sea robins could be seen clearly within

that distance moving over the bottom of the harbor. The calling is heard at night, as well as in the daytime. In a few instances during the summer, production of the staccato call was heard to follow various sharp percussive sounds—the discharge of a high-energy spark into the water, the explosion of a detonating cap in the harbor, and the slamming of the live car lid on the raft.

Since the first staccato calls of 1955 were heard from fishes caged at the surface, while sea robins characteristically feed on the bottom, it was thought that warming of surface waters might have initiated calling from surface specimens earlier than their calling would ordinarily have commenced. However, temperatures taken with a bathythermograph during July and August of 1955 showed that there was a thorough mixing of water over the 72-foot depth under the raft, and no records obtained showed over a two-degree F. variation in temperature from the surface to the bottom.

While the significance of the calling behavior of the sea robin to its survival and normal behavior is as yet undetermined, the observations reported have demonstrated that sound is significant to the behavior of sea robins. The work has demonstrated that it is possible to control sound production by these marine fishes with man-made sounds. The findings stand as an exception to the general rule (Moulton and Backus, 1955) that production of man-made sounds causes only quickened swimming movements of fishes.

It should also be of interest to students of marine animal noises that it is possible to incite, without handling or trapping, the calling of marine fishes by transmission of appropriate signals, thus making it possible to move experiments to the natural environment from the confines of laboratory tanks, which under the best of conditions suppress and may otherwise modify calling behavior.

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Summary

1. A vibrant grunt and a staccato call of sea robins in the Woods Hole area are described. Sounds similar to these can be obtained by manipulation of the air bladder and by stimulation of the nerves to the drumming muscles.

2. It is suggested that the sea robin grunt is part of a general alarm reaction, and that the staccato call is related to the breeding behavior of the sea robin. It is suggested that the staccato call may serve as a species recognition device in waters where visibility is relatively poor.

3. A method of controlling production of the staccato call is described. Production of the call can be initiated by playing into the water imitations of the call and recordings of the call itself. The calling can be suppressed by playing of signals of 200 to 600 cps, and, less consistently, by playing of signals of 600 cps to 2 kc.

4. The results obtained furnish an exception to the general rule that sound production causes only quickened swimming movements of free fishes, and demonstrate the possibility of exercising some degree of control over the behavior of fishes in nature with man-made sounds.

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CYTOLOGICAL EVIDENCE FOR A ROLE OF THE CORPUSCLES OF STANNIUS IN THE OSMOREGULATION OF TELEOSTS[1]

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Until recently there was no experimental evidence to show what tissue in teleosts was responsible for elaboration of the vital hormones of the adrenal cortex. For many years adrenal cortical function was attributed to the corpuscles of Stannius first described by Stannius in 1839. This was largely because of their morphological position on the ventral surface of the kidney, analogous to the adrenal position in other species of the vertebrate series, and because they showed histological characteristics of endocrine function. Although Giacomini (1908), in studies based on histology and morphology, attributed adrenal cortical function to secretory epithelium lining the cardinal veins, he did not relinquish the corpuscles of Stannius as a part of the adrenal complex but rather designated them as the posterior interrenal tissue. He called the glandular tissue, which is associated with the cardinal veins in the head kidney, the anterior interrenal tissue.

Many important factors have mediated against considering the corpuscles of Stannius as true adrenal tissue. Pettit (1896) demonstrated compensatory hypertrophy of one corpuscle after removal of the other in eels. However, Vincent (1898) claimed to have extirpated both corpuscles in eels without causing death to result. The inference is that if the glands were as vital in the physiology of the teleost as the adrenals are in the mammal, the eels would have been unable to survive without them. Garrett (1942) confirmed previous observations of Giacomini (1911) that the corpuscles originate embryologically from evaginations of the pronephric ducts and not from mesothelium which provides the adrenal cortical anlagen of other vertebrates. In certain forms, as *Amia,* Garrett thought that the glands might also arise from mesonephric tubules. Rasquin (1951) showed that the corpuscles were not stimulated by implantation of fresh carp pituitary or injection of mammalian ACTH as was the anterior interrenal tissue. Pickford (1953) confirmed the fact that the corpuscles were not under pituitary control by demonstrating that there was no atrophy of the glands after hypophysectomy in the marine cyprinodont *Fundulus heteroclitus,* although this investigator was also unable to find any effect of hypophysectomy on the anterior interrenal tissue. However, Chavin (1954) reported complete atrophy of anterior interrenal after hypophysectomy in the goldfish and no reaction of the corpuscles of Stannius to the same operation.

Rasquin (1951) reported that lipids were not demonstrated in anterior interrenal cells of *Astyanax* by the use of osmic acid or Sudan IV techniques. However, further investigation with more modern techniques applied to paraffin rather than frozen sections has shown that this is not the case. The use of Baker's acid

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hematein stain with acridine red, as suggested by Rennels (1953), has shown a positive reaction for phospholipids in the anterior interrenal tissue of the teleost. The diffuse nature of this tissue and the fact that patches of cells containing positive droplets alternate with those that are negative in reaction make it possible to lose positively reacting tissue in broken-up frozen sections. The discovery that the glandular cells of the corpuscles of Stannius also contained phospholipid granules provided a technique for studying the cellular reaction of the gland to various experimental procedures.

Materials and Methods

A total of 135 individuals of the species *Astyanax mexicanus* (Filippi) were used in the course of the experiment. All were sexually mature, between one and two years of age and appeared in healthy and vigorous condition. Experimental procedures involved the injections of water, electrolytes, DCA and pitressin. Table I shows the distribution of fish among the various procedures and the times allowed to elapse between injection and killing. In each group of three or more animals, the tissues from one fish were fixed in Bouin's fluid and stained in Harris' hematoxylin and eosin; the tissues from the remaining fish in each group were fixed in calcium-formol and stained with acid hematein (Baker, 1946). The fish were killed by decapitation and the musculature from one side of the body and the air bladder were removed before placing the body in the fixing fluid. About an hour later the kidneys, containing the corpuscles of Stannius, were dissected out and returned to fresh fluid. This procedure insured rapid fixation of the rather labile granules of the glandular tissue. All tissues were imbedded in paraffin and sectioned at five microns and some were counterstained with acridine red.

The volume of all fluid injections was 0.05 cc. except for those of pitressin-and-water, where 0.15 cc. was used and the injections were made into the abdominal cavity. Glass-distilled water was used, alone and for dissolving sodium and potassium chloride. The implantation of dry DCA pellets was also made intraperitoneally. These contained 75 mg. each, and, inasmuch as this amount was far too great for the small fish, the pellets were broken up and small pieces were used. With this method there is no way of measuring the amount of hormone absorbed by any one fish. However, pieces of pellet were observed in all implanted fish at the time of death, indicating a continuous supply of hormone throughout the experimental period.

Two series of injections were made with pitressin for a study of the reaction to antidiuretic hormone. The first of these consisted of one pressor unit in 0.05 cc. aqueous solution in each fish. The second series consisted of the same amount of hormone diluted to 0.15 cc. with glass-distilled water, for the purpose of giving an additional stimulus of water load in the fish.

Weights of *Astyanax* of this age group range between one and two and one-half grams. Weighing the fish either before or after killing was avoided, first because prompt fixation was necessary, and secondly, because the fright caused by extra handling might have had some effect on granulation in the cells to be studied.

In addition, one *Astyanax* was used for each of the following methods: the pyridine extraction test (Baker, 1946) to verify the phospholipid content of the tissues reacting positively to acid hematein, Cowdry's modification of Bensley's

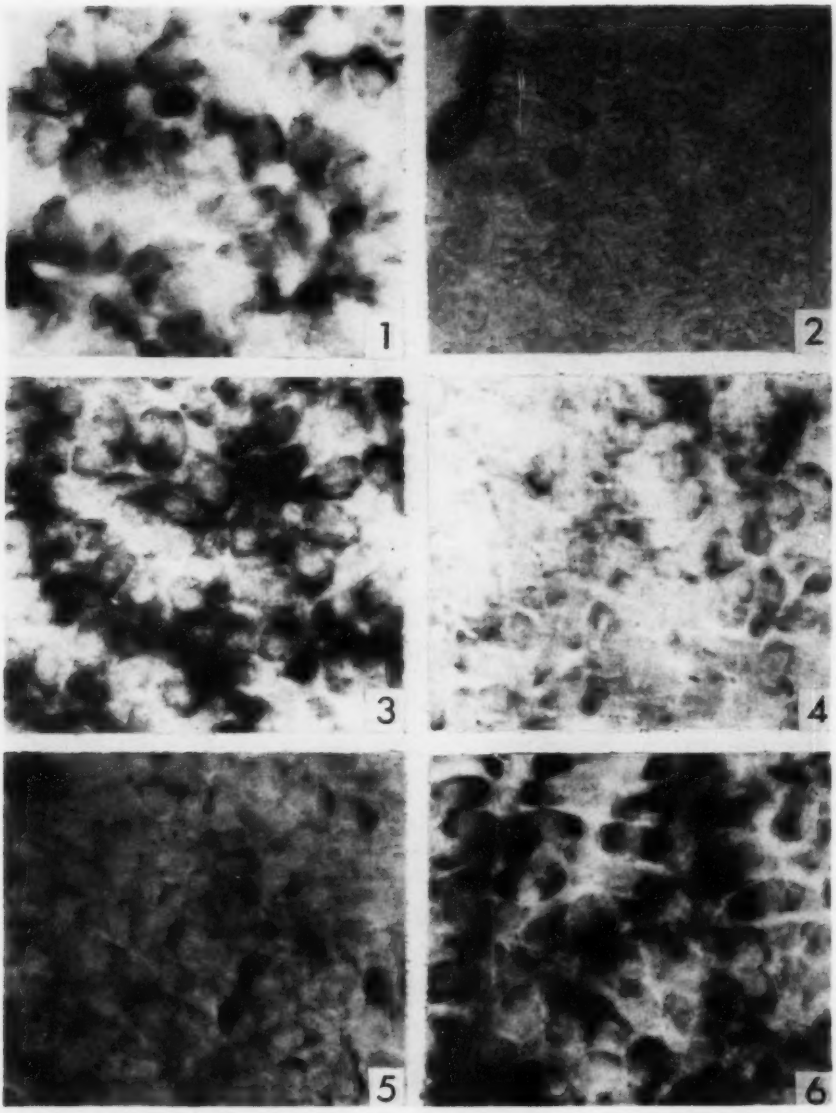
TABLE I

Numbers of Astyanax used and duration of experimental procedures

Experimental procedure	Nos. of fish	No. of days before sacrifice
Implantation of DCA pellets	3	1
	3	3
	3	5
	3	8
	3	10
	3	18
	3	25
	6	75
Daily injections 0.05 cc. distilled water	3	1
	3	3
	3	5
	4	7
Daily injections 2.0 mg. sodium chloride	3	1
	3	3
	3	5
	3	7
Daily injections 0.5 mg. potassium chloride	3	1
	3	3
	3	5
	3	7
Single injection 1.0 mg. potassium chloride	3	30 minutes
	3	1 hour
	1	2 hours
	4	24 hours
Single injection aqueous pitressin 1 unit	3	30 minutes
	3	1 hour
	3	2 hours
	3	4 hours
	3	6 hours
	3	24 hours
Single injection 1 unit pitressin plus 0.1 cc. water	3	30 minutes
	3	1 hour
	3	2 hours
	3	4 hours
	3	6 hours
	3	24 hours
Tests for pyridin extraction, mitochondria, and ascorbic acid	4	

method for mitochondria as given by Jones (1950) to ascertain the nature of the granules in the cells of the corpuscles of Stannius, and Bourne's (1936) method to discover the presence or absence of ascorbic acid in the same glands.

Lastly, ten fish were injected with 0.1 cc. distilled water five days a week for four weeks and ten others were allowed to live in 1% sodium chloride for ten days. The corpuscles of Stannius of all these were studied after staining with Baker's acid hematein.



Cells of the corpuscles of Stannius of *Astyanax mexicanus* stained with Baker's acid hematein after various experimental procedures. Magnification 1200 ×.

FIGURE 1. Normal untreated fish showing blackened granules in the cytoplasm.

FIGURE 2. Cells unstained after pyridin extraction test, indicating the blackened granules to be composed of phospholipid.

Experimental Results

Many teleost tissues reacted positively to Baker's acid hematein stain: red blood cells, myelin sheaths of nerves, zymogen granules in exocrine pancreas, granules in cells of both anterior interrenal and corpuscles of Stannius, and granules of the coarse granular eosinophiles found in the connective tissues and sometimes in the blood of teleosts. The only tissues that remained positive after pyridin extraction were the erythrocytes and some of the large granules in the anterior interrenal cells.

Figure 1 is a photomicrograph showing the positive reaction to Baker's acid hematein stain in the cells of the corpuscle of Stannius of a normal, untreated *Astyanax*. The black material is made up of phospholipid granules and possibly also mitochondria. Figure 2 shows the corpuscle cells devoid of any stained granulation after application of the pyridin extraction test; the dark stained objects are erythrocytes. The corpuscle is normally made up of small granular cells that are greater both in amount of cytoplasm and size of nucleus at the periphery than at the center of the gland. Sometimes the gland has a cord-like appearance caused by two lines of cells on either side of a capillary. The nuclei are distal to the blood vessel, and the cytoplasmic granules crowded into the part of the cell adjacent to the capillary wall. At other times, probably associated with less activity, no cord-like or acinar arrangement can be detected and the cells appear to be crowded within the confines of the connective tissue capsule without any obvious architecture. Bobin (1949), using Sudan Black B and osmic acid, has also demonstrated the lipid nature of the cellular granules of the corpuscles in the European eel. In this species, she was able to distinguish both mitochondria, which were rod-like or slightly filamentous, and secretory granules, which were spherical. A similar distinction was not apparent in *Astyanax*. When stained for mitochondria, the cells were found to be crowded with these organelles which were spherical and smaller than the granules stained with acid hematein. The size difference, however, may be an artifact related to the different fixing and staining process. The probability is that acid hematein stains both types of inclusions at the same time. After the corpuscle cells are degranulated by experimental procedures there is a simultaneous loss of so much cytoplasm that mere non-reactivity of mitochondria cannot be responsible for the loss of staining reaction. The application of acid silver nitrate for demonstration of ascorbic acid resulted in only very rare stained granules in occasional corpuscle cells. However, Fontaine and Hatey (1955) have found a high content of ascorbic acid in these glands in the salmon.

Effects of desoxycorticosterone acetate (DCA)

Implantation of DCA pellets brought about an enlargement of the cells of the corpuscles with a simultaneous increase in number and size of cytoplasmic granules.

Figure 3. Increase in granulation in corpuscle cells of a fish that had received injections of water five days a week for four weeks.

Figure 4. Decrease in granulation in corpuscle cells of a fish that had lived in 1% saline for ten days.

Figure 5. Decrease in granulation 6 hours after injection of one unit undiluted aqueous pitressin.

Figure 6. Increase in granulation 6 hours after injection of one unit pitressin plus an added water load.

The hypertrophy of the cells with their heavy granulation, particularly at the periphery of the glands, was observed as early as three days after implantation. After 18 days, heavy granulation was seen in all the cells throughout the gland. At the same time, the cord-like arrangement of the cellular elements along the capillaries was pronounced, particularly noticeable under the low power of the microscope. This reaction was maintained throughout the 75-day period. The hypophyses of the three animals killed 18 days after implantation were sectioned and stained with Masson's trichrome stain. Histological study revealed that these glands were apparently normal in every detail. Prolonged administration of DCA had no such effect on the transitional lobes as was observed by Rasquin and Atz (1952) after injection of cortisone in the same species. Administration of cortisone brought about an inversion of the ratio of acidophils to basophils with subsequent marked acidophilia of the lobe.

Effects of water

The same results in the cells of the corpuscles of Stannius, enlargement and heavy granulation, were obtained by injections of distilled water. However, study of the glands on the first and third days after injections were started showed an initial shrinkage of the cells, causing spaces to occur between them, and there was some evidence of degranulation on the first day. From the fifth day onward the cells were hypertrophied and heavily granulated. The granulations were evident even in the hematoxylin and eosin-stained sections where they were markedly acidophilic. Figure 3 is a photomicrograph of the corpuscle of a fish injected five days a week for four weeks with distilled water. Heavy granulation is very evident here. Furthermore, hypertrophy of the entire gland was seen in most of the ten fish subjected to this procedure; sometimes the hypertrophy occurred in only one corpuscle so that the hypertrophied organ would be twice the size of the other one in the same animal.

Effects of sodium chloride

Continued sodium chloride injection at a dosage of 2 mg. per day brought about only slight hypertrophy of the cells of the corpuscles of Stannius and granulation appeared about the same as that seen in normal glands. However, the glands in the fish that lived 10 days in 1% saline showed degranulation of the cells. This reaction is seen in Figure 4.

Effects of potassium chloride

Because of the toxicity of potassium chloride the daily dose had to be reduced to 0.5 mg. in order to ensure survival. Doses of one mg. each were fatal, the fish dying between two and 24 hours after injection. Some of these were preserved for study (Table I). After one injection of 0.5 mg., the cells of the Stannius corpuscles appeared large and heavily granulated. Subsequently degranulation occurred and the cells were much smaller in size. In addition, the cord-like arrangement of the cells was disrupted and red blood cells were scarce as a result of decreased blood supply. Degranulation was obvious in all fish dead 24 hours after the one-mg. dose. In sections stained with hematoxylin and eosin, it could be

plainly seen that the degranulation resulted in considerable loss of cytoplasm from the cells. Nuclei were crowded together, especially in the center of the gland where they were virtually denuded of cytoplasm. In the corpuscles of the fish receiving the smaller daily doses complete degranulation was not seen; some glands contained more stained granules than others but in general, all the cells were smaller than normal and the granulation was fine and usually confined to a small area about the nucleus.

Effects of pitressin

The cells of the corpuscles of Stannius reacted differently to the two procedures employed for pitressin administration. With pitressin alone, the cells were degranulated and decreased in size although this was not so extreme as when potassium chloride was used. One-half hour after injection, the cells showed a very fine granulation distributed mostly in a narrow ring around the nucleus. The same picture was obtained after one hour except that the hematoxylin and eosin-stained sections showed the nuclei to be somewhat shrunken and hyperchromatic. After two hours the granules seemed larger and more numerous and this slightly heavier granulation persisted up to six hours after injection. By 24 hours, however, the gland had returned to its normal appearance. Figure 5 represents the corpuscle cells six hours after injection of pitressin.

In great contrast to Figure 5 is Figure 6 which represents the corpuscle cells six hours after the injection of diluted pitressin. The hypertrophied cells with heavy black granulation were typical of all the corpuscles from one to six hours after injection. After only one-half hour the cells appeared small and granulation was fine and confined mainly to a ring around the nucleus, as described for the injections of pitressin alone. After 24 hours, the corpuscle, although still heavily granulated, had begun to take on a more normal, lighter stained appearance.

All the experimental procedures, with the exception of pitressin injections, served to decrease the staining response of mitochondria in kidney tubules. In the case of DCA administration, the staining reactivity returned to the mitochondria of the tubules after 75 days, indicating that the fish had made some physiological adjustment to long continued administration of this hormone. The kidney tubules of all fish included in the pitressin-injected group showed deeply stained mitochondria, especially noticeable in the more distal parts of the tubules, the intermediate segments and the ureters.

Discussion

Much of the literature pertaining to the corpuscles of Stannius is now of historical interest only. A full bibliography up to 1946 was published by Aboim. The most recent contribution is by Bauchot (1953) who studied the comparative anatomy of the glands in 47 different species including both marine and fresh water forms, attempting to relate their anatomical location to phylogeny. He concluded that the most primitive position of the corpuscles is an anterior one about midway of the length of the kidney, and the most evolved, a posterior one, much nearer the vent, although there were exceptions, as in the salmonids and *Solea* where the location of the corpuscles was not compatible with the systematic position of

the fishes. This author also considered the number of corpuscles to have a phylogenetic significance. Thus the holostean, *Amia*, possesses between 40 and 50 corpuscles and the salmonids anywhere from six to 14, while the usual number for most teleosts is two. In *Astyanax* the number was found to vary between two and four, although two was by far the most common. Garrett (1942) also thought the large number of corpuscles was a more primitive condition, the advanced condition of two major corpuscles being produced by the fusion of many smaller ones. Garrett, after demonstrating the origin of the corpuscles from the pronephric duct, suggested a homology of the glands with a part of the Mullerian duct and Bauchot is in agreement with this suggestion. Some of the reasoning behind this idea is concerned with the fact that the chondrosteans, in which the corpuscles of Stannius are absent, have reduced and non-functional Mullerian ducts, while the holosteans and teleosteans, in which there are remnants of the Mullerian ducts, possess the corpuscles of Stannius.

In general, two kinds of changes were brought about in the cells of the corpuscles by the experimental procedures. Degranulation, loss of cytoplasm and consequent decrease in size of cells accompanied the administration of potassium chloride, undiluted pitressin, and long-continued immersion in saline. Hypertrophy of cells with increase in numbers and size of blackened granules accompanied the administration of water, diluted pitressin and DCA. The non-reactivity of the cells after sodium chloride injection may be owing to the fact that the dosage was too small to have an effect. Unfortunately, little is known about the action of DCA in fish.

Final interpretation of these results must await further study, particularly by investigators who have physiological techniques at their disposal. It is possible that the corpuscles were responding merely to the increased water load, that degranulation after administration of potassium chloride was owing to the toxic effects of the potassium ion and that the degranulation after undiluted pitressin was an initial release of secretion unaccompanied by further immediate stimulation. It seems fairly obvious from these results that the corpuscles of Stannius have some function in osmoregulation, inasmuch as changes in the granulation are accompanied by changes in the metabolic activity of the kidney tubules.

If the function of the corpuscles has to do with water excretion it might help to explain why various investigators have been unable to demonstrate water retention in teleosts after administration of posterior lobe hormones. Burgess, Harvey and Marshall (1933) were unable to demonstrate any effect on urine flow in the catfish, *Ameiurus nebulosus*, with 0.2 to 2.0 units of pitressin per kilogram. Their graph shows a slight increase in water diuresis for the catfish after pitressin injection, probably without statistical significance. Boyd and Dingwall (1939), using pituitrin, were unable to cause an increase in weight in young carp, although comparable doses of the hormone acted positively on frogs to increase the weight as a consequence of water retention. Fontaine and Raffy (1950) thought that the failure might have been due to the use of mammalian hormone and therefore they repeated the experiments with preparations made from the pituitaries of fish, carp, eels, etc. Their fish posterior pituitary preparations proved to be potent in causing water retention in frogs, but negative results were still obtained in the fish.

Callamand *et al.* (1951) reported that the hypophysis was not concerned with osmoregulation in eels inasmuch as they were able to place hypophysectomized

Anguilla back and forth from fresh water to sea water and even into water with twice the salinity of sea water without any deleterious effects. On the other hand, Pickford (1953) found that hypophysectomized *Fundulus heteroclitus* were unable to survive in fresh water or diluted sea water, although this species is normally euryhaline. Burden (1956) was able to keep these fish alive in fresh water by replacement therapy of *Fundulus* pituitary material. He postulated the secretion of an unknown factor by the *Fundulus* pituitary which regulates the salt balance of the fish in fresh water. Other investigators (Matthews, 1933; Abramowitz, 1937) have reported no difficulty maintaining hypophysectomized *Fundulus* in fresh water.

Neurosecretory material in the hypophysis and hypothalamus of a teleost was first described by Scharrer (1932). Since then Arvy, Fontaine and Gabe (1954) have shown that neurosecretory material in the hypothalamo-hypophyseal systems of *Phoxinus* and *Anguilla* can be depleted by subjecting the fish to hypertonic solutions, indicating a sensitivity of the neurosecretory apparatus to the need for retaining water in the internal environment. Rasquin and Stoll (1955) have shown that neurosecretion may be withheld in the brain nuclei after injection of pitressin, indicating a reaction to the antidiuretic principle, even though it has not yet been demonstrated physiologically.

Interpretations of cellular activity in the corpuscles of Stannius for this report depend mainly on the reaction of the cells to Baker's acid hematein stain for phospholipids. Unfortunately the significance of phospholipin in cellular metabolism is not yet thoroughly understood. Among several theories reviewed by Sinclair (1934), one considers that phospholipids are increased during cellular activity, particularly in actively secreting glands such as the salivary glands and the corpus luteum. Rennels (1953) also believes that phospholipids play an important role in the secretory activity, citing the staining reaction of hypophyseal acidophils, adrenal cortical cells and mitochondria. He points out that different phases of activity of both secretory granules and mitochondria are accompanied by positive or negative reactions to the stain. After gonadectomy, mitochondria of the delta cells of the rat hypophysis showed an increased activity presumably associated with increased secretory function of the cells, even though the secretory granules of these cells have no affinity for the stain.

Cain and Harrison (1950) have also suggested that histochemically demonstrable phospholipid is connected with some special metabolic activity. In a cytological study of the adrenal cortical cells in the rat, they have shown that mitochondria have an affinity for acid hematein during the phase of active secretion in the cell, and that after discharge of secretory products, the mitochondria become negative to the stain. The mitochondria become positive to the stain before the lipid droplets, but the droplets are not formed from the mitochondria; rather they are separate and distinct within the cytoplasm. Therefore, for the present report, the increase in positive staining response of the corpuscles of Stannius has been interpreted as an indication of increased metabolic activity. This interpretation is strengthened by simultaneous hypertrophy of the cells and of the whole organ with increased stainable granulation.

All these results strongly indicate the presence of a special mechanism antagonistic to the antidiuretic hormone in teleosts, and this may possibly be produced by the corpuscles of Stannius which are not found in other vertebrates.

SUMMARY

1. The effects of DCA, pitressin, water, and sodium and potassium chloride on the cytology of the corpuscles of Stannius were studied by means of Baker's acid hematein stain for phospholipids. The fresh water characin *Astyanax mexicanus* was used.

2. Two kinds of changes in the cells of the corpuscles were brought about by the experimental procedures: degranulation, loss of cytoplasm and consequent decrease in size of cells accompanied the administration of potassium chloride, undiluted pitressin and long continued immersion in 1% sodium chloride, and hypertrophy of cells with increase in numbers and size of blackened granules accompanied the administration of water, diluted pitressin and DCA.

3. Loss of staining reaction in mitochondria of kidney tubules was associated with increased secretory activity in the corpuscles of Stannius except in the case of long continued DCA administration and administration of pitressin.

4. The results are interpreted as indicating a function of the corpuscles of Stannius in the osmoregulation of these fish possibly connected with excretion of excess water.

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ON THE ECOLOGY OF THE LOWER MARINE FUNGI [1, 2]

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The lower marine fungi (*i.e.*, Myxomycetes and aquatic Phycomycetes) have generally been described as occurring on plant and animal hosts. While several forms have been described as saprophytes, the only genus known to occur on debris is *Labyrinthula* (Sparrow, 1936). All other described species are endobiotic or epibiotic with rhizoids penetrating living or dead host cells. They are also sporadic in occurrence. The application of a semi-quantitative plating technique to sea water has now established that lower fungi are far more common in littoral waters than previous studies indicate, and has suggested a new ecological niche for these fungi.

The plating technique which we used consisted of spreading samples of sea water with a bent glass rod on the surface of a solid isolation medium (Table I).

TABLE I

Isolation Medium

Sea water	80 ml./100 ml.
Gelatin hydrolysate	0.1%
Glucose (added aseptically)	0.1%
Liver 1:20	0.001%
B-vitamins	
Agar	1.5%
	adjusted to pH 7.5

Marine mineral base (Vishniac, 1955) was sometimes substituted for sea water. Gelatin hydrolysate and the B-vitamin mixture were prepared as by Vishniac and Watson (1953). Liver extract concentrate 1:20 was obtained from the Nutritional Biochemicals Co.

The moisture content of the medium is critical: the agar plates should be dried overnight, but not allowed to stand for more than two days. Just before use the plates are spread with 2000 units of Penicillin G (Squibb, buffered) and 0.5 mg. of dihydrostreptomycin sulfate (Wyeth) in concentrated aqueous solution. These plates will then absorb a 0.2-ml. sea water sample in an hour or two. After inoculation, the plates were incubated at 20 degrees or less. Such plates support the growth of lower fungi, yeasts, molds, and some diatoms, but few or no bacteria. We have found unfortunately incomplete suppression of bacterial growth when water samples taken from City Point, New Haven, near the sewage disposal plant,

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were plated. Colonies of lower fungi visible to the naked eye appear in a week or ten days. Colonies were counted at 30 × and further examined at 100 ×.

The results of spreading triplicate 0.2-ml. samples of sea water taken in June, 1956 from waters in and around Woods Hole, Mass. are given in Table II. The number of species represented is probably a minimum figure, since only colonies which were markedly distinct in color, texture, or size of thallus were picked for further study. Yeasts, molds and diatoms were not counted regularly. Yeasts were rare. From 0–5 colonies of molds, mainly *Penicillium* spp. of uncertain provenance, were found on those plates for which molds were counted. The presence of molds was not correlated with the presence of lower fungi.

It is evident from the poor agreement between triplicate platings in Table II that this technique is quantitative only within an order of magnitude. The procedure suffers from the following defects:

TABLE II

Occurrence of lower fungi in sea water

Origin of sample	Colonies/plate	No. species
1. Sea water tap, W.H.O.I.	0, 0, 0	0
2. Great Harbor	0, 0, 0	0
3. Eel Pond	94, 34, 20	7
Tap water, from bowls with algae		
4. Algae washed ca. 5 hrs.	23, 8, 20	5
5. Algae washed ca. 24 hrs. (another collection)	15, 10, 1	6
Water expressed from algae		
6. Pilings, U. S. Fish and Wildlife Station	62, 5, 56	2
7. Rocks, Red Spindle (Grassy Island)	66, 214, 35	7

(1) Spreading the sample, necessitated by the aerobic nature and poor temperature tolerance of the desired forms, entails the loss of 3 to 8% of a 0.2-ml. sample. The amount of sample remaining on the glass rod was determined by weighing the salt remaining on the glass rod after spreading a 20% NaCl solution.

(2) Either a thallus or a spore may give rise to a colony. The ecologic implications of the presence of a thallus or a spore in the sample are quite different.

(3) A thallus may produce zoospores in the interval between spreading and drying of the sample, giving rise to several colonies. The occasional appearance of groups of colonies of the same form on a plate was presumed to have this cause.

(4) Not every viable spore or thallus present in the sample may give rise to a colony. The conditions provided for growth were chosen after study of a limited number of marine forms—members of the genera *Labyrinthula, Sirolpidium, Thraustochytrium,* and three unidentified isolates. Obviously, forms with other requirements may exist. We have been particularly interested in assessing the probable extent of the selective action of the medium.

It is probable that forms with additional nutritional requirements would grow sufficiently on the medium used to form a countable colony. The ability of an individual organism, previously well nourished, to form a colony in the absence of required nutrients is inversely proportional to the quantity of nutrient required. Generally, absence of a suitable carbon source is felt first, amino acids required as

growth factors next, and lastly the absence of added vitamins—some of which may be stored in quantities sufficient for many generations. It is highly improbable that suitable carbon sources for any of the fungi with which we were concerned were lacking. As a source of amino acids, gelatin hydrolysate is inferior, since it is poor in methionine and in the aromatic amino acids. Nevertheless, we have maintained methionine-requiring Phycomycetes on gelatin hydrolysate media for over a year of semi-monthly transfers. The feasibility of isolating forms requiring growth factors not present in this medium was demonstrated by the isolation of sterol-requiring *Labyrinthulas* from two of the sea water samples used in this study (4 and 7, Table II). Syntrophism may occur (though no obvious examples were seen) on these plates.

An experimental approach to this problem was made by plating a sample of sea water from *Ulva* colonies on pilings at City Point, New Haven, in triplicate on the isolation medium, on the isolation medium without liver extract, on the isolation medium without liver extract, vitamins, or glucose, and on a medium containing glucose, glutamate, and thiamine as its only organic constituents. At the same time isolates known to require liver extract and known to require glucose, amino acids, and vitamins were plated on these media. Of the media used, only the glucose-glutamate-thiamine agar gave significantly lower sea water counts. This medium also failed to support typical colony formation by isolates known to require growth factors. The isolates requiring liver extract failed to form typical colonies on the medium from which liver, glucose, and vitamins were omitted also, though the omission of liver extract alone did not affect their growth under the conditions used.

It is, on the other hand, quite possible that fungi exist which did not form recognizable colonies because they were inhibited by the ingredients of the medium. Representative colonies were picked from the isolation plates made at Woods Hole into tubes of semi-solid (0.1% agar) isolation medium for further study. It then developed that, in semi-solid media, each of the organic constituents of the medium was somewhat inhibitory to some of the fungi isolated. The results of the comparative plating of City Point sea water outlined above and of similarly plating isolates known to be inhibited by ingredients of the isolation medium indicated that, for these fungi, the inhibitions are relieved by growth on a solid agar surface. The validity of this conclusion must be restricted to the fungi examined.

In principle, these defects are not unique to our procedure; they require restatement here because this is the first application of plating techniques to the lower marine fungi, indeed to ecologic studies of any aquatic Phycomycetes, and because they bear on the conclusions to be drawn from our results. The results of Table II suggest, first, that the presence of these fungi is correlated with the organic content of the water examined, since the highly polluted Eel Pond is richer than Great Harbor (on the incoming tide). Second, in Great Harbor (and in the Hole), fungi are associated with algae: they may be isolated from water taken from finger bowls in which algae were being kept under a constant drip of previously fungus-free tap water. They may be isolated from water squeezed by hand from masses of attached algae growing on rocks and pilings.

The organic content of polluted waters, such as those of the Eel Pond, could reasonably be expected to support a population of free-living non-filamentous fungal saprophytes, just as of bacterial saprophytes. We have calculated, from cell counts of representative cultures, that the amount of soluble organic material required to produce a single thallus of the common holocarpic or monocentric marine Phycomy-

cetes is of the order of 1 mμ g. But the development of techniques for establishing directly the presence of a free-living fungus population would be very desirable. Two instances of association with organic debris were noted. One colony of a monocentric Phycomycete was found on a plate on a stray grain of pine pollen. One species (an undescribed myxoid form here referred to as isolate "S"), of which 1–22 colonies were found on every plate containing lower fungi, formed colonies which were as often as not centered on a microscopic bit of nondescript organic debris.

In view of the known endo- and epibiotic habit of marine fungi, the apparent association with algae required further investigation. The algae with which the sea water samples of Table II were associated were examined microscopically for the presence of fungal thalli. As might have been expected from the experience

TABLE III

Fungi associated with algal surfaces

	No. fragments	No. species fungi/fragment
1. Algae from rocks at Red Spindle (Grassy Island)		
Ectocarpus	1	2
Antithamnion	8	1–3
Polysiphonia	4	1–3
Ceramium	3	1–3
2. Algae from rocks of Pine Island		
Ectocarpus	4	1–2
Elachistea	5	1
Punctaria	2	2
Chorda filum	1	3
Callithamnion	18	1–3
Antithamnion	4	2
	1	0
Polysiphonia	3	0
Bryopsis	10	1–2
	4	0

of previous investigators (see Petersen, 1905; Sparrow, 1934, 1936; Kobayashi and Ookubo, 1953), recognizable thalli were rare. The one species which Sparrow (1936) found epidemic later in the summer—*Ectrogella perforans* in *Licmophora*—was conspicuously absent. The only form found during these examinations was *Petersenia lobata* (?) on *Polysiphonia urceolata* (*fide* W. R. Taylor) collected at the Red Spindle and allowed to rest for 12 days in a finger bowl under dripping sea water (as suggested by Sparrow). Five days later the infection had disappeared.

The results of plating small pieces of algae showed, in marked contrast, that the algal surface not contaminated by lower fungi was rare. Bits of algae, usually strands approximately one cm. long, were cut from the collected plants, drawn gently over the edge of a petri dish to remove water and placed on the surface of the usual antibiotic-isolation medium agar. No fungal thalli (possibly because of their small size) could be recognized at 100 × on the algal fragments at the time of plating. Neither fungal thalli nor rhizoids were seen within the cells of these algae at any time, though the specimens were examined at 430 × at the end of the incubation period. The association of lower fungi with fragments of algae collected at the Red Spindle (Grassy Island) in Great Harbor and at Pine Island

(off Nonamesset Island, in the Hole) is shown in Table III. Molds and yeasts were observed fairly frequently; specimens in this series which were obscured by such forms have been omitted from the tabulation.

Are these fungi on the algal surface or in the surface film of water surrounding the algal fragment? If the surface film of water is, at a guess, about 0.01 ml., one would expect to find of the order of 1–10 viable units of lower fungi in it. The results of Table III are not inconsistent with this estimate, but are unfortunately not quantitative, because discrete colonies are rarely formed around algal fragments. But we are inclined to consider, for two reasons, that the fungi found in association with algal fragments resemble many of the marine bacteria in being present, as thalli, on the surface of the alga. First, we have attempted (in a limited number of trials) to wash the fungi away with sterile sea water. The results, even with such algae as *Bryopsis plumosa* and *Polysiphonia urceolata* which were free of the forest of hairs and epiphytes found on most marine algae, were poor. No more fragments were free of fungi after washing than before, and essentially the same type of fungi was present. Secondly, although both holocarpic and eucarpic Phycomycetes were among the forms associated with algal surfaces, the predominant form was, as in sea water samples, the myxoid surface-loving "S." Every algal fragment listed in Table III as having associated fungi had "S" associated with it. The association of "S" with bits of organic debris has already been noted. "S" was also found to be associated with two of six copepods plated as part of a rather unproductive plankton haul from Great Harbor. Another surface-loving form, *Labyrinthula* sp., occurred less frequently on *Polysiphonia*, *Ceramium*, and *Ectocarpus*.

Conclusions and Summary

Our data therefore suggest that the lower marine fungi occupy essentially the same ecologic niche as the marine saprophytic bacteria. These fungi can be found in suitably polluted sea water in numbers of the order of 1–500,000 viable units/liter but less than 5000/liter in more open waters of Woods Hole. The fungus count increases, as has long been noted for bacteria (Gazert, 1906), in the presence of macroscopic algae. They also resemble marine bacteria in their association with surfaces. As a group, these fungi differ from marine bacteria in being strongly aerobic. One may justifiably wonder as to the basis of their success in competition with bacteria in this niche. Studies, now in progress, of the individual species of fungi concerned may clarify this question.

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THE EFFECT AND AFTER-EFFECT OF VARIED EXPOSURE TO LIGHT ON CHICKEN DEVELOPMENT

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The basic factor necessary for initiating development of the gonads of birds, according to Rowan (1938), appears to be length of day. Intensity above a certain low threshold, he says, appears to be of no significance. Frequency of light stimulation plays an important role according to Benoit (1936) and, more recently, Staffe (1951), Kirkpatrick and Leopold (1952), and Farner *et al.* (1953a). On the other hand, with uninterrupted lighting early growth was delayed and reproductive performance adversely affected, as shown by studies of Lamoreux (1943), Callenbach *et al.* (1944), and Ringrose and Potter (1953).

Since most of the studies cited above involved wild birds, it remains to be seen whether their results are directly applicable to the domestic fowl; this is particularly true for strains of chickens that have been bred for egg production. Previous work on the effect of intermittent lighting on laying hens by Wilson and Abplanalp (1956) has indicated that egg production can be maintained with very small amounts of light energy, provided it is given intermittently (less than six one-minute intervals in 24 hours). These results tend to support earlier findings by Staffe (1951), who demonstrated that short light flashes from 1500-watt lamps were effective in stimulating laying hens to increase winter egg production.

The present study was conducted in order to gain further information about the effects of intermittent lighting upon the development of chickens. It was, however, restricted to an investigation of early growth and the onset of sexual maturity in pullets.

Material and Methods

Two experiments were conducted with Single Comb White Leghorn stock of the University of California at Davis. In the first, pedigreed chicks were hatched on December 4, 1953. The purpose of the test was to study the effect of supplementary light on the development of chickens. Each hen's chicks were distributed as equally as possible among six experimental groups. Each lot of chicks was then brooded and reared in 15 × 15-foot pens of a house with open fronts. Natural light was given to all six pens, but three received supplementary artificial light from a continuously burning 100-watt incandescent bulb. Three brooding methods were used with the two light treatments as follows:

Group	Brooder type	Light regime
1	4 infrared lamps	Natural light only
2	Electric	Natural light only
3	Gas	Natural light only
4	4 infrared lamps	Natural light + 100 W continuous
5	Electric	Natural light + 100 W continuous
6	Gas	Natural light + 100 W continuous

The gas brooders provided a somewhat higher room temperature than either of the other types.

Brooding was discontinued when the chicks were 6 weeks old. A week later, at 7 weeks of age, the chicks were scored for feathering. Four grades were used in assessing completeness of feathering, ranging from 1 for poorest feathering to 4 for best performance. Both sexes were scored.

At the same time a few males from large families were killed in order to determine comb and testis weights. Body weight was measured first at 7 weeks of age and from then on at 4-week intervals. Age at first egg and the average weight of the first three eggs were determined whenever possible.

The second experiment was to determine effects of intensity and frequency of lighting upon growth and sexual development of pullets. Pullet chicks were hatched on July 1, 1955, and all brooded alike up to 5 weeks of age. The following brooding and lighting regime was used:

0–1 weeks of age: 10-watt bulb; continuous light
1–3 weeks of age: 40-watt bulb; continuous light
3–5 weeks of age: 10-watt bulb; continuous light

Shielded incandescent light bulbs were the only source of heat.

At 5 weeks of age, and in some cases again at 90 days of age, the experimental lots of birds were subjected to changes in lighting regime, according to the following plan:

Group	Light intensity (foot-candles)		Total hrs. light in 24 hours	Number of light periods in 24 hours after 90 days of age
	35–90 days	After 90 days		
A	0.5–30.0	0.5–30.0	14.0	1
B	0.0–0.4	0.5–30.0	14.0	1
C	0.0–0.4	0.0–0.4	1.5	12; 6; 3; 2.
D	0.5–30.0	0.0–0.4	1.5	12; 6; 3; 2.
E	0.5–30.0	0.4–6.6	1.5	12; 6; 3; 2.

As indicated in the table above, groups C, D, and E were each divided into four subgroups at 90 days of age, and the latter were subjected to light periods of varying frequency, while the total duration of lighting was held constant. The 12 light periods per 24 hours consisted of 7.5 minutes each, followed by 52.5 minutes of darkness. Similar regularly spaced periods of light were used where six and three stimuli were given. In the case of two light periods, however, 45 minutes of light was alternately followed by 7 hours, 15 minutes and by 15 hours, 15 minutes of darkness. Group A served as control and was held under "cool white" fluorescent lighting with 14 hours of light per day. Light intensities varied between 0.5 and 30.0 foot-candles according to the location of individual laying cages.

Four windowless climatic chambers were used for this experiment; they have been described in detail by Wilson and Abplanalp (1956). Each chamber was subdivided into two sections with a partition of black sisalkraft, in order to allow replication of treatments. Thus, each lighting regime was given in two different chambers. A diagrammatic outline of this arrangement is given in Figure 1.

Temperatures were held constantly at approximately 80° F. The pullets were

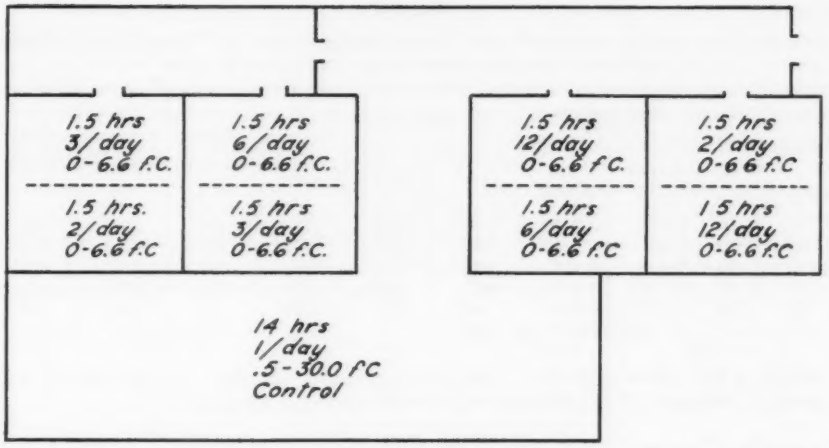

FIGURE 1. Floor plan of experimental rooms showing location of replications. Total light per day, number of light periods per 24 hours, and intensity in foot-candles are given in order for each subgroup.

placed in individual 10-inch cages when they were 5 weeks old. Two double rows of cages were arranged in step fashion, which meant that the birds in the upper rows were somewhat closer to the light source than those in the lower cages. The top rows were 18 inches from the light source, while the bottom ones were 3 feet distant. Light intensities varied between 0.0 and 0.4 foot-candles for the lower tiers and between 0.4 and 6.6 foot-candles for the upper ones.

Experimental lighting was held at suboptimal intensities in order to bring out more clearly the possible effects of frequency of light periods.

The following traits were observed and analyzed: 1) Body weights at 12, 20, and 28 weeks of age; 2) age at first egg for individual pullets; and 3) average

TABLE I

Effect of supplementary light on growth, feathering and sexual development of December-hatched chicks.
Number of individuals in parenthesis

C-22	Body weight, females gms.				Testes wt. at 7 weeks (gms.)	Feather score	Aver. age at 1st egg (days)	Aver. wt. 1st 3 eggs (gms.)
	7 wk.	12 wk.	16 wk.	20 wk.				
No artificial lights	(189) 436	(189) 1023	(189) 1322	(189) 1605	(36) .240	(553) 3.47	(189) 157.7	41.0
100-watt lights cont.	(190) 452	(190) 1006	(190) 1303	(190) 1519	(32) .284	(545) 3.21	(190) 163.8	43.6
Difference	−16*	17	19	86**	−.044**	.26**	−6.1*	−2.6**

* = $P < .05$.
** = $P < .01$.

TABLE II

Relation of light intensity at different stages of growth and age at first egg. Group A and B received 14 hours of light after 90 days of age, other groups 1½ hours

Group	No.	Light treatment foot-candles		Body weight (gms.)			Median age at 1st egg (days)	Aver. wt. 1st 3 eggs (gms.)	At 248 days % maturity
		Between 35–90 days	After 90 days	12 wk.	20 wk.	28 wk.			
A	10	0.5–30.	0.5–30.	1031.	1514.	1744.	153.	39.7	100
B	10	0–0.4	0.5–30.	990.	1505.	1779.	161.	42.2	100
C	40	0–0.4	0–0.4	1017.	1391.	1707.	191.	48.9	85
D	40	0.5–30.	0–0.4	1013.	1466.	1838.	179.	47.5	72
E	40	0.5–30.	0.4–6.6	958.	1426.	1705.	169.	45.1	95

weight of first three eggs laid (whenever possible). Median age at first egg was used as a measure of sexual maturity of entire groups of pullets.

RESULTS

The results of the first test are shown in Table I. Continuous light added to natural illumination apparently favors early growth of chicks up to at least 7 weeks of age. Later on, and most conspicuously at 20 weeks of age, the effects of continuous light supplement are just the opposite. Significantly higher body weights were found at 20 weeks for the birds brooded under natural light only.

Comb weight of 7-week-old males shows little or no effect as the result of different light treatments, but testis weights responded in the same way as body weight. Added continuous lighting resulted in significantly heavier testes at 7 weeks of age.

Continuous light tends to retard feathering and sexual maturity of pullets (but not of males). The 6-day differences in maturity between supplemented and control groups was highly significant. This delay in age at first egg was associated with a somewhat higher weight of first eggs.

The results of the second experiment are given in Tables II and III. In Table II the data are arranged according to total amount of daily lighting and light intensities. Table III, on the other hand, shows the effects of increasing frequencies of light periods with a given amount of light applied after 90 days of age.

TABLE III

Frequency of light intervals/24 hrs. in relation to growth and age at first egg

Light foot-candles	Light periods/24 hrs.		Total light/24 hrs.	No.	Aver. body weight (gms.)			Median age at 1st egg (days)	Aver. wt. 1st 3 eggs (gms.)
	Number	Length			12 wk.	20 wk.	28 wk.		
0–6.6	12	7½ min.	90 min.	30	991.	1437.	1751.	168.0	46.5
0–6.6	6	15 min.	90 min.	30	980.	1410.	1736.	174.0	46.1
0–6.6	3	30 min.	90 min.	30	1011.	1481.	1826.	190.5	48.0
0–6.6	2	45 min.	90 min.	30	1002.	1381.	1750.	189.0	47.6
0.5–30.	1	14 hrs.	14 hrs.	20	1010.	1510.	1759.	160.0	41.2

The observed body weights of pullets do not show any clear-cut effects of either light intensity, amount of light, or lighting frequency.

Sexual maturity as measured by median age at first egg, however, was strongly affected by differences in lighting, both before and after 90 days of age. The control treatment (group A) with highest light intensity during early and late periods of development matured earliest. Group B, with low light intensity to 90 days of age but high intensity thereafter, matured 8 days later. Similarly, groups E, D, and C show consistently adverse effects of reduced light intensities upon age at first egg.

The influence of light intensity on maturity is apparently operative over a considerable period of early development and is not merely restricted to a period very close to the onset of egg production. This can best be shown by regrouping the results of median age at sexual maturity.

Light before 90 days of age	Light after 90 days of age		
	Dim	Bright	Difference
Dim	191	161	30
Bright	179	153	26
Difference	12	8	—

Here it may be noted that bright light administered before pullets were 90 days old advanced sexual maturity by only 12 and 8 days, while intensive lighting after 90 days of age produced effects of 30 and 26 days. This clearly indicates that the influence of light intensity upon sexual maturity becomes more pronounced the closer toward onset of lay it can operate.

Table III shows the effects of more frequent light periods on age at first egg. In each case the birds received a total of 90 minutes of dim light in 24 hours. The results are clear-cut and show that light is more effective in stimulating sexual development the more frequently it is applied. Thus, 12 short periods of dim light permitted pullets to mature almost as early as one 14-hour period of intensive light. The same amount of dim light given in only two doses, on the other hand, resulted in extremely late maturity. These findings are shown more clearly in Figure 2. The data represent medians for the replicate lots. Each lot contained subgroups which differed in light intensity. See regime for groups C, D and E.

Discussion

The first experiment confirmed that growth of the domestic fowl can be influenced by light. All-night lights may have aided early growth in this experiment by providing the chicks more opportunity to feed. Frequent feedings may be particularly helpful when the crop capacity of chicks is still poorly developed.

The adverse effects of continuous lighting after 7 weeks of age, as found in this study, are in agreement with findings by Tomhave (1954).

The results of the second test with respect to growth after 12 weeks of age are not conclusive. They fail to substantiate reports by Clegg and Sanford (1951) and by Barott and Pringle (1951), who found that intermittent lighting has a beneficial effect on early growth of chickens prior to 12 weeks.

In this study it has been found that continuous light has adverse effects on feathering, becoming more serious as the birds become older. Similar effects of continuous light on turkeys have been reported by Mueller *et al.*, 1951.

The role of continuous light in the sexual development of chicks appears to be a complex one. While the 7-week-old cockerels showed increased testes weight under continuous lighting, the pullets appeared to be delayed in their development. Unfortunately, there were no testes measurements available for cockerels near maturity; hence, it is difficult to assess the possible importance of refractory behavior of pullets. Evidence of retarded growth at 20 weeks of age indicates, however, that both cockerels and pullets may have been delayed sexually near the point of maturity.

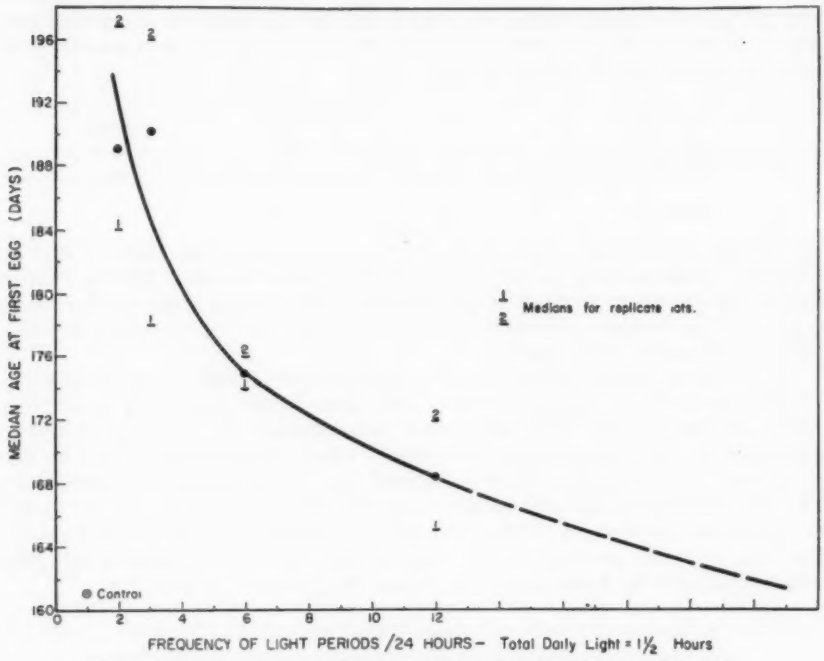

FIGURE 2. The relation of frequency of light periods and age at first egg.

From the results of the second experiment, as well as from an earlier report by Wilson and Abplanalp (1956), it is clear that the total amount of light is not the sole determinant in stimulating sexual development of pullets. Aside from the adverse effects of continuous lighting (which may possibly be due to a nervous fatigue of the animal), it has also been demonstrated here that light intensity and the distribution and frequency of light stimuli are important variables that must be considered in the problem of light stimulation.

The present results show that under limiting conditions of low light intensities and short periods of light exposure, the rate of sexual development increases with longer total daily exposure to light, greater light intensity, and more frequent stimulation. These relationships may not hold when either light intensity or total duration of light exposure is increased beyond certain thresholds. Nevertheless, they

permit certain conclusions of practical value. Thus, where natural light is to be supplemented, it would seem reasonable to use frequent but short intervals of artificial lighting in place of continuous lighting or of light periods adjoining the natural day.

Several attempts have been made recently to rationalize the response of birds to light. Wolfson (1953) interprets his data as supporting the hypothesis that the total daily dose of light determines the response. He postulates, however, that the proportion of light exposure to darkness in a given cycle is the critical factor in determining the response rather than the daily dose of light.

Kirkpatrick and Leopold (1952), in agreement with Jenner and Engels (1952), maintain that the dark period *per se* is a major controlling factor in the response of birds to light. Such an interpretation appears primarily different in terminology and emphasis but seems to add little to an understanding of the problem. It has been criticized by Hammond (1953) and by Farner *et al.* (1953b).

In the light of this study, the theory advanced by Farner *et al.* (1953b) seems the simplest and most suitable for explaining the action of light in reproduction of birds. In brief, it postulates that there exists a light-sensitive gonadotropic mechanism capable of activation almost immediately upon onset of lighting; it remains active throughout the light period and even for some time following termination of the latter. This theory has helped to explain the effectiveness of extremely short photoperiods (a total of 6 minutes in 24 hours) in maintaining egg production of chickens (Wilson and Abplanalp, 1956). We believe this theory suitable to explain the current findings which show the increasing effectiveness of a given amount of light when given in numerous small doses.

The formulation of general theories on the basis of published evidence is seriously hampered by non-uniformity in experimental material, procedures, and terminology. On the basis of Farner's theory and present findings, one may attempt to interpret light response by means of three independent main effects and their interactions, namely:

1. Total daily amount of light exposure
2. Light intensity
3. Frequency of light intervals.

Additional assumptions are needed in order to explain refractoriness and perhaps seasonal changes in reproduction of birds, but the present experiments do not permit any new interpretation of their role.

Summary

The present experiments were designed to determine the effects of:

a. Total daily amount of light
b. Intensity of light
c. Frequency of light intervals

on the growth and development of Leghorn chickens.

a. *Total daily amount of light exposures:* The first test dealt with the effect of supplementing natural light with continuous light. Continuous light improved body weight of all birds and testes size of males at 7 weeks, but impaired feather development. Continuous lighting delayed sexual maturity of pullets, and growth in both sexes was retarded until they reached 20 weeks of age.

In the second experiment under suboptimal light intensities, light exposure has no effect on body size after 12 weeks of age. Rate of sexual development was increased by larger daily exposures to light. These effects were more pronounced when treatments were applied to pullets over 90 days old than during earlier stages of development.

b. *Intensity of light:* Three light intensities were applied to growing birds: 0.0–0.4 foot-candles, 0.4–6.6 foot-candles, and 0.5–30.0 foot-candles. Growth was not affected by lower light intensities, but sexual maturity was delayed.

c. *Frequency of light intervals:* Body weight was unaffected by lighting frequency. Sexual maturity, however, was significantly advanced when suboptimal light exposure and light intensities were applied in frequent but small doses. Thus, it was found that 12 periods of 7.5 minutes of dim light per day produced a rate of sexual development almost equal to the rate with 14 hours per day of normal lighting. These results are taken as further proof that the after-effects of light on the reproductive mechanisms of chickens are considerable

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